DEVELOPMENTAL AND MOLECULAR ASPECTS OF TURION FORMATION IN *SPIRODELA POLYRRHIZA* AND ITS INDUCTION BY ABSCISIC ACID

by

CHERYL CHRISTINE SMART

DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH

1981

VOLUME I

I declare that this thesis was composed by myself and that the work presented herein, unless otherwise stated, is my own.

September, 1981

ACKNOWLEDGEMENTS

I should firstly like to express my gratitude to my supervisor Dr. A.J. Trewavas for all his help and his infernal optimism. Special thanks also go to Dr. A.E.S. Macklon of the Macaulay Institute who gave me invaluable support enabling me to carry out the compartmental analysis, and to all the people at the Macaulay who made my short stays at the Plant Physiology Department such a pleasure, especially Dr. P.C. DeKock, Mr. A. Hall and Mr. H. Shepherd.

I am also grateful to all the members of the Botany Department, Edinburgh who have helped me during the 3 years, especially Dr. C.J. Leaver, Dr. J.E. Dale, Miss H. Quinn, Mrs. P. Docking, Mr. W. Foster, Mrs. M. Waugh, Mr. D. Denham, Mr. P. Kelly, Mr. C. McKay and all in the EM suite and the workshop. I should also like to thank Prof. M. M. Yeoman for allowing me to continue my work until Christmas 1980.

I acknowledge receipt of an instant award from the Science Research Council.

CONTENTS

ABBREVIATIONS

A	- ampere(s)
mA	- milliampere(s)
ABA	- abscisic acid
Ac	- acetate
AFS	- apoplastic free space
AMPS	- ammonium persulphate
ASA	- American Standards Association (film speed)
ATP	- adenosine 5'- triphosphate
ATPase	- adenosine 5'- triphosphatase
azetidine	- L-azetidine-2-carboxylic acid
bis-acrylamide	- NN'methylenebisacrylamide
BSA	- bovine serum albumin
Butyl PBD	- 2-(4'-tert-butylphenyl)-5-(4"-biphenyl)-1,3-4-oxadiazole.
^{o}C	- degrees Celsius
cet. bromide	- cetyltrimethylammonium bromide
Ci	- Curie
mCi	- millicurie
μCi	- microcurie
cpm	- counts per minute
DFS	- Donnan free space
DTT	- dithiothreitol
DNA	- deoxyribonucleic acid
e	- base of natural logarithm = 2·71828
EDTA	- ethylenediaminetetra-acetic acid
EEO	- electroendosmosis

ER	- endoplasmic reticulum
F1	- Fraction 1 protein (RuBP carboxylase)
Fig.	- figure
g	- gram
mg	- milligram
µg	- microgram
ng	- nanogram
GA	- gibberellin
GA_3	- gibberellic acid
g_{av}	- unit of gravitational force (average)
g_{max}	- unit of gravitational force (maximum)
GTP	- guanosine 5'-triphosphate
h	- hour
H/2	- half-strength Hutner's medium
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IEF	- isoelectric focusing
l	- litre
ml	- millilitre
µl	- microlitre
log	- logarithm to the base 10
m	- metre
cm	- centimetre
cm^2	- square centimetre
cm^3	- cubic centimetre
mm	- millimetre
µm	- micrometre

nm	- nanometre
M	- molar
mM	- millimolar
μM	- micromolar
min	- minute(s)
MO	- 2-methoxyethanol
mole	- mole
mmole	- millimole
μmole	- micromole
pmole	- picomole
N	- normal
NAD	- β-nicotinamide adenine dinucleotide
NADP	- β-nicotinamide adenine dinucleotide phosphate
no.	- number
NP-40	- Nonidet P40
OD	- optical density
o	- degrees
p.	- page
PAGE	- polyacrylamide gel electrophoresis
PAL	- phenylalanine ammonia lyase
PAS	- 4-aminosalicylic acid, sodium salt
PCA	- perchloric acid
PEP	- phospho(enol) pyruvate
PGO	- peroxidase/glucose oxidase
pH	- negative common logarithm of the molar concentration of hydrogen ions
pI	- isoelectric point

poly A - polymer of adenosine

p.s.i. - pounds per square inch

r - correlation coefficient

RNA - ribonucleic acid

mRNA - messenger ribonucleic acid

rRNA - ribosomal ribonucleic acid

tRNA - transfer ribonucleic acid

RNAase - ribonuclease

RuBP - ribulose 1,5-bisphoshate

s - second(s)

SDS - sodium dodecyl sulphate

SE - standard error of the mean

S30 - supernatant of a 30,000 g_{max} centrifugation

TCA - trichloroacetic acid

TEMED - NNN'N' tetramethylethylenediamine

TNS - tri-iso-propylnaphthalenesulfonic acid, sodium salt

Tris - tris(hydroxymethyl)aminomethane

UV - ultraviolet

v/v - volume percent

V - volt

W - watt

w/v - weight percent

WFS - water free space

5FU - 5-fluorouracil

8HQ - 8-hydroxyquinoline

2ME - 2-mercaptoethanol

ABSTRACT

The ability of S. polyrrhiza to form turions in response to ABA, made it an ideal model for an investigation into the development of the dormant state and its regulation by ABA. Addition of ABA to a culture of S. polyrrhiza resulted in growth inhibition at concentrations as low as 10^{-9}M, growth being completely arrested at 10^{-5}M. However, over a single order of magnitude range around 10^{-7}M, ABA also induced the production of turions.

The morphological changes and structure of the vegetative frond and turion were characterised at both the light and electron microscope level, and the cellular differences between frond and turion cells investigated by detailed stereological analysis. It was found that only primordia $\leqslant 0{\cdot}7$ mm long could be induced by ABA to develop into turions. During the development of a cell, it acquires ABA sensitivity for turion formation, and is only in this state for approximately 14 hours. Ultrastructural analysis showed that the cells within this sensitivity window are still actively dividing, and the developmental switch-over to rapid cell expansion and separation is believed to mark the end of the cell's sensitivity to ABA.

The turion of S. polyrrhiza is characterised by its small size, reniform shape and dark brown colouration. The mesophyll is undifferentiated and totally lacking the substantial aerenchyma development found in the vegetative frond. The cells of the turion, while reaching approximately the same final size as the vegetative frond cells, accumulate numerous starch grains, thick cell walls and large deposits of tannins and anthocyanin pigment, at the expense of vacuolar expansion.

A comprehensive examination and evaluation of the biochemical parameters associated with turion formation was carried out. The developmental process leading to the formation of turions was accompanied by a repression of nucleic acid and protein synthesis and an enhancement of secondary metabolism. DNA synthesis in the developing turion was inhibited within 3 hours of ABA addition, followed by a repression of protein(especially soluble protein) synthesis after 24 hours, while RNA synthesis was not inhibited until 3 days. The inhibitory effect of ABA on protein synthesis was found to be selective and the synthesis of several novel proteins appeared to be induced.

The relationship between the changes in the polypeptide and mRNA profiles during the development of the turion was investigated. The rapid general inhibition of protein synthesis at early stages of turion formation could not be accounted for by the levels of translatable mRNA, indicating an effect of ABA at the translational level. The specific alteration to the pattern of *in vivo* labelled proteins could however have resulted from control of the level of specific mRNAs for those particular proteins. Only after 3 days in ABA, when the developing primordium is committed to the turion developmental pathway, is there a total inhibition in the production of mRNA leading to the shut-down of all primary processes and the onset of the irreversible events leading to the dormant state.

No obvious ion effects causal to turion formation or initiation could be detected by compartmental analysis, although the ionic fluxes and concentrations of the mature turion were very different to those of the vegetative frond. Thus it was concluded that the

mechanism of action of ABA in responsive tissue is the regulation of gene expression and the production of specific mRNAs; followed by a sequence of steps leading to inhibition of DNA, protein and RNA synthesis, inhibition of cell division, expansion and differentiation, and an enhancement of secondary metabolism.

The molecular basis of the differential sensitivity to ABA which was found throughout this work is discussed in terms of receptor mechanisms.

CHAPTER 1

INTRODUCTION

1.1 DEVELOPMENT AND DORMANCY

The phenomenon of plant or animal development remains one of the most challenging aspects of modern biology, representing an area in which there is considerable scope for a 'breakthrough' comparable in stature to the advances achieved in biochemistry and molecular biology.

In its broadest sense 'development' applies to that whole series of changes through which a given organism will evolve during its entire life cycle. During normal vegetative growth plants continually adapt their development in order to cope with any stress inducing or adverse changes in the environment. Under extreme conditions in which the survival of the plant itself may be at risk, immediate growth and development can be suspended and a state of dormancy assumed.

Given the widest interpretation, dormancy refers to any temporary cessation of active growth. The dormant state that results from unfavourable conditions such as low temperature or water stress, and which is reversed upon the plant's experiencing more favourable environmental conditions is defined as 'imposed dormancy'. 'Innate dormancy' refers to a state of temporarily suspended growth that is directly attributable to some internal stimuli, and is characterised by growth remaining suspended even under external conditions which apparently favour growth.

Dormancy is exhibited by many seeds, the buds of a wide variety of storage organs, the terminal buds of temperate woody perennials and of tropical trees which exhibit episodic growth, the flower buds

of tropical trees and by the turions of several aquatic plants.

As with all developmental processes in plants, dormancy is believed to result as a consequence of plant growth regulator activity(ies), in particular the growth regulatory sesquiterpenoid abscisic acid (ABA). The role of ABA as a possible initiator and/or regulator of bud dormancy has and is currently receiving a great deal of attention. The aquatic plant Spirodela polyrrhiza (a member of the Lemnaceae) will undergo dormant bud (turion) formation under a variety of conditions. The ability of this plant to form turions in response to exogenous ABA, made it an ideal model for studies aimed at understanding development, dormancy, and the role of ABA in the regulation of these processes.

In addition to a general discussion on bud dormancy and its possible regulation by ABA, this introduction contains a detailed description on the structure and vegetative growth of the Lemnaceae. This afforded the necessary background of information from which a discussion on turion formation could proceed. A review of the literature on those aspects of ABA that are relevant to dormancy precedes an outline of the aims and approach adopted in this thesis.

1.2 BUD DORMANCY

The most extensively studied examples of bud dormancy are those observed in the buds of temperate woody perennials, which is associated with the survival of the vegetative shoot apices during the winter months. In temperate woody plants dormant buds are formed typically in late summer or autumn, and the whole tree or shrub enters a dormant phase. Even though some temperate woody

species (e.g. Betula pubescens and Larix decidua) can be maintained in continuous growth for 18 months or longer under long day conditions in a warm greenhouse, eventually all temperate species form dormant buds even under environmental conditions evidently optimum for growth (Wareing and Phillips, 1970).

One of the most important factors affecting and controlling the induction of bud dormancy in temperate woody plants is day length. In the majority of woody plants studied so far, long days promote vegetative growth and short days bring about the cessation of extension growth and the onset of bud dormancy (Wareing, 1956).

Emergence from dormancy of perennial plants of temperate regions generally is conditioned by low temperature. Most woody plants need to be exposed to a period of winter chilling to overcome dormancy. Although chilling is necessary to break dormancy of the buds, warm temperatures are necessary for the actual resumption of growth of the buds (bud burst) after the chilling period.

There is very little information on the molecular events leading to the formation of dormant buds. However, it has been suggested that a highly specific block to metabolism is a characteristic feature of innately dormant buds, at least at certain stages in their development. Tuan and Bonner (1964) compared the rates of RNA synthesis by dormant and non-dormant potato tuber buds and the capacity of the chromatin from such buds to support DNA-dependent RNA synthesis. They found that dormant buds possessed only a very limited capacity for both DNA and DNA-dependent RNA synthesis, as did the isolated chromatin from the dormant buds.

Similar results were reported by Jarvis et al. (1968) using intact tissue and isolated chromatin from dormant and non-dormant embryos of Corylus avellana. These authors concluded that the DNA is completely repressed in the dormant cell. While dormant unchilled embryos of pear had much lower level of RNA synthesis than chilled embryos, the repression of RNA synthesis was found to be associated with a decreased chromatin bound polymerase activity. In this instance the possiblity of reduced template availability being additionally involved was not ruled out (Khan, 1972).

1.3 BUD DORMANCY AND ABSCISIC ACID

The idea that the regulation of dormancy may involve growth inhibitory substances was first put forward on the basis of Hemberg's observations on dormancy in potato tubers and the buds of Fraxinus (Hemberg, 1949a, 1949b). An attempt to determine the nature of the endogenous inhibitors in leaves and buds of sycamore (Acer pseudoplatanus) (Robinson and Wareing, 1964; Phillips and Wareing, 1959), led ultimately to the isolation and identification of abscisic acid in extracts of this material (Cornforth et al., 1965).

Eagles and Wareing (1964) had been able to induce the formation of buds with many of the features of resting buds on birch (Betula pubescens) seedlings grown under long day conditions by treating the plants with a partially purified extract of plants grown under short days. When synthetic ABA became available, El-Antably et al. (1967) reported that repeated applications of ABA to the leaves and apices of Betula pubescens grown under long days caused the formation of

resting terminal buds. These authors also induced dormant winter bud formation in Ribes nigrum and Acer pseudoplatanus. However, subsequent attempts to induce bud dormancy in various woody species by the application of ABA have been unsuccessful, although apical growth was inhibited (Perry and Hellmers, 1973; Saunders et al., 1974; Hocking and Hillman, 1975).

In many woody species the formation of dormant buds is promoted by short days and it was shown that under these conditions the leaves exert an inhibitory effect upon the shoot apices which appears to bring about the formation of dormant buds (Wareing, 1954). This observation suggested that under short day conditions increased amounts of endogenous ABA are produced in, and exported from leaves, early measurements of inhibitor levels in these tissues appearing to support this hypothesis (Phillips and Wareing, 1959). Other authors have compared ABA levels in the shoot tips of a variety of species under short and long photoperiods, by using physical methods of ABA detection (Lenton et al., 1972; Powell, 1976; Alvim et al., 1976, 1979). However, in no case did they find that ABA levels were higher in plants grown under short days compared with those exposed to long day conditions.

Various studies have shown that dormant buds often contain relatively high concentration of ABA (During and Bachmann, 1975; Harrison and Saunders, 1975; Wright, 1975; Emmerson and Powell, 1978). Other studies however, have failed to show any consistent correlation between ABA levels and the degree of dormancy in flower buds (Corgan and Martin, 1971) or seeds (Dennis et al., 1978; Berrie

et al., 1979).

Thus attempts to establish the role of ABA in the bud dormancy of woody plants have given inconsistent results. The same cannot be said for the role of ABA in the induction of the turion of Spirodela polyrrhiza, a much simpler and more accessible system.

1.4 THE LEMNACEAE AS EXPERIMENTAL ORGANISMS

Members of the Lemnaceae (duckweeds) possess many features which make them good experimental organisms. These plants can be grown rapidly on simple inorganic media using aseptic tecniques (Clark, 1930; Saeger, 1930). Control of environmental factors can be achieved, since both individuals and groups of plants can be manipulated easily. Moreover, all species can be maintained indefinitely by vegetative reproduction, thus eliminating genetic variation. The small size and relatively simple structure of the Lemnaceae, compared with other angiosperms, makes them ideal for investigators interested in whole plant physiology. It is therefore not surprising that an increasing number of workers are using members of the Lemnaceae for physiological and biochemical research.

A review of the experimental and descriptive literature on the Lemnaceae is given by Hillman (1961). More recent major areas of interest include the action of growth regulators (Tasseron-de Jong and Veldstra, 1971; McCombs and Ralph, 1972; McLaren and Smith, 1977; Sankhla and Huber, 1979; Hartung et al., 1980), flowering (Wołek, 1974b; Tanaka and Takimoto, 1977; Scharfelter et al., 1978), enzyme activity and regulation (Knypl, 1977; Stewart and Rhodes, 1977; Gordon and Koukkari, 1978; Tobin and Suttie, 1980),

photoperiodism and phytochrome responses (Rombach and Spruit, 1968; Hillman, 1975, 1976; Loppert et al., 1978; Rombach, 1978), photosynthesis (Lindeman, 1973; Witzum et al., 1979), respiration (Hillman, 1977; Morris and Barker, 1977), mineral nutrition and uptake processes (Fraser, 1974; DeKock et al., 1978; Knypl, 1978; Kondo and Tsudzuki, 1978; Loppert, 1979), ultrastructural studies (Anderson et al., 1973; Wroblewski, 1973; Chui and Falk, 1975; Melaragno and Walsh, 1976; Pan and Chen, 1976), protein and nucleic acid metabolism (Trewavas, 1972a; Davies and Humphrey, 1978; Tobin, 1978; Weinbaum et al., 1979), secondary metabolism (Reznik and Menschick, 1969; Elliott, 1977; Zennie and McClure, 1977; Krause and Strack, 1979) and ecological studies (Wołek, 1974a; Landolt, 1975; McLay, 1976).

1.5 SYSTEMATICS

The Lemnaceae are aquatic plants which float on the surface of fresh still water. They are the smallest angiosperms and each plant, which is never more than 10 mm long, is called a 'frond' since its derivation is uncertain.

The Lemnaceae are divided into four genera - Spirodela, Lemna, Wolffiella and Wolffia (Hegelmaier, 1895; Lawalrée, 1945). Spirodela and Lemna fronds are more or less flat, ovate and leafy in appearance. Most Spirodela species have 2 or more roots per frond while Lemna only has one. Wolffiella and Wolffia have no roots and are thalloid (Lawalrée, 1943).

Most taxonomists have placed the Lemnaceae in the monocotyledonous order Arales, their close relationship to the Araceae being

traced through the type species of this family, Pistia stratiotes L., the water lettuce (Daubs, 1965). As early as 1868, Hegelmaier had accepted this relationship based on the gross morphological similarities of Pistia and Spirodela. Arber (1919) set down these similarities in detail. Brooks (1940) concluded that the Lemnaceae were derived from a degenerate Pistia-like ancestor and that they show a reduced structure, forming a reduction series from Spirodela, through Lemna, to Wolffiella, down to Wolffia.

1.6 GENERAL FROND STRUCTURE AND GROWTH

Spirodela and Lemna produce daughter fronds from 2 meristematic regions contained within pockets on each side of the narrower end of the mother frond, near the point of attachment of the roots, or node (cf. Rimon, 1964). This end of the mother frond is called the proximal end, the other wider end being termed distal. Each daughter frond also reproduces in this way while still attached to the mother, due to a poor abscission mechanism (Witzum, 1974b). In this way, the fronds form small colonies or plantlets. The fronds of Wolffiella and Wolffia only have one reproductive pocket (Brunaud, 1974b) and in Wolffiella, the mother and several generations of daughters may remain attached, forming large wheel-like colonies. In all four genera, each mother frond is capable of producing many daughters in its lifetime, the ultimate number being dependent upon the environmental conditions.

In Spirodela and Lemna, daughter fronds are formed alternately from side to side (Brunaud, 1974a; Guern, 1965; Vintejoux, 1969),

developing earlier in one pocket than the other. Clones of the same species differ as to which pocket is plus(+) (i.e. producing the first daughter, but this usually remains constant in a given clone (de Sloover, 1966; Witzum, 1979). Detailed investigations on the development of daughter fronds in Spirodela oligorrhiza were made by Rimon (1964) and Rimon and Galun (1968). They found that a daughter frond just visibly protruding from the mother frond, already had 2 reproductive pockets, each with a daughter frond of its own. Moreover, these young "buds", which are the third generation of fronds when counted from the mother frond, themselves contained 2 tiny pockets, each containing a bud primordium. The authors proposed the following developmental scheme:
(1) in a bud consisting entirely of meristematic tissue, and with a length of about 18 cells, two pockets can be discerned. A new bud primordium is initiated shortly after. (2) Further differentiation starts when the bud reaches 30 cells in length, and the typical frond structure first becomes apparent at the distal end. (3) After a certain amount of development, a meristematic area is retained at the node, where a root is initiated (S. oligorrhiza only has one root per frond). (4) The daughter frond continues to grow by cell elongation of the distal two-thirds and by cell division at the node. (5) The daughter frond bursts the pocket and is now visible, growing mainly by cell expansion, while cell division continues in the proximal part between the node and the stolon (or stalk) which connects it to the mother frond. Cell differentiation continues by the formation of aerenchyma and vascular bundles. These authors also describe the formation of a "lobe-like scale" in the axil

between the mother and daughter frond, which is of course the axillary bud (Witzum, 1974b), which develops when the first daughter has abscised.

When flowering occurs, it is always initiated in the minus(-) pocket (Hegelmaier, 1868; Kandeler, 1955; Landolt, 1957). This asymmetry is already determined in the embryo, but as shown by hybrid analysis, is not determined by either nulear or cytoplasmic genes (Kasinov and Kasinova, 1974).

In most species flowering is very unusual. Each flower consists of one pistil and 2 stamens. These organs are enclosed in a membranous spathe which is ruptured at maturity (Hegelmaier, 1895; de Sloover, 1966; Maheshwari and Kapil, 1963). Flowers in Wolffiella and Wolffia only have a single stamen and no spathe. The flower arises from the upper side of a floating frond (Mason, 1938; Maheshwari, 1954).

1.7 GENERAL FROND ANATOMY

The main part of the mother frond is composed of aerenchyma i.e. parenchyma cells separated by large intercellular air spaces, which gives the frond its buoyant nature. The upper epidermis is cutinised and is water repellent, and contains stomata which remain open all the time (Wagner, 1973). Sculthorpe (1967) reports that both the upper and lower epidermis are rich in chloroplasts. However, in a detailed study of S. polyrrhiza, both the upper and lower epidermis were observed to be "composed of small cells lacking contents" (Jacobs, 1947).

Melaragno (1974) reports that in Lemna minor, the upper

epidermis is composed of cells containing a thin layer of cytoplasm in which an occasional chloroplast is found. The guard cells appeared to be completely devoid of cytoplasm. This author noted that the cells of the lower epidermis were slightly larger than the cells of the upper epidermis, but were never exposed to an intercellular air space, unlike the cells of the upper epidermis. Jacobs (1947) reported guard cells with chloroplasts in Spirodela polyrrhiza, and this finding was confirmed by Le Pabic (1972) in an ultrastructural study of the same species. Wolffia arrhiza has guard cells which contain dense cytoplasm (Anderson et al., 1973).

Both Spirodela and Lemna fronds show reduced vascular tissue and it is generally accepted that no vascular tissue is present in the other genera. Lemna trisulca has been shown to contain bundles with one xylem element above and one sieve element below, with 2 companion cells (Arber, 1920). Jacobs (1947) found the opposite arrangement in Spirodela polyrrhiza. In Lemna minor there is a single tracheary element, 2 - 3 sieve elements and their companion cells, and between 5 and 10 small vascular parenchyma cells which separate the bundle from the large air spaces. The xylem is located dorsally and the phloem ventrally (cf. Jacobs, 1947).

In Spirodela and Lemna, a large bundle runs from the node to the proximal end of the frond and is in fact free of the frond at this end in Spirodela. This is the stalk or stolon which attaches the young frond to its mother during its development. At the node, several bundles diverge into the distal end of the frond, forming veins. There are 3 veins in Lemna minor and 5 or more in Spirodela polyrrhiza (Brooks, 1940; Jacobs, 1947).

The roots are regarded as adventitious, and form at the node just beneath the lower epidermis. Sometimes the ruptured epidermis forms a short sheath at the top of the root, and at the tip of the root is a root cap. The roots are less than 0•5 mm in diameter and their length varies with environmental conditions. The root cortex is compsed of 3 - 4 layers of large chloroplast containing parenchyma cells, surrounded by a single layer of epidermal cells with only a thin layer of cytoplasm and no chloroplasts (Melaragno, 1974). The root chloroplasts are photosynthetically active (Pirson and Göllner, 1953). At the junctions of the inner rings of cortical cells is a regular pattern of air spaces. The innermost ring of cortical cells, the endodermis, surrounds the vascular tissue, which consists of a ring of 10 - 12 phloem cells surrounding a single tracheary element. On opposite sides of this element is a regular arrangement of 2 sieve elements and 2 companion cells. Arber (1920) suggests that the root's function is to keep the plant in an upright position and to keep the plants in tangled masses to give protection against fast moving water. The roots certainly have no root hairs, the whole surface of the frond being able to absorb nutrients from the medium.

1.8 TURION STRUCTURE AND ANATOMY

Many aquatic angiosperms produce specialised buds which are important both in vegetative reproduction and in the survival of the species through periods of stress e.g. winter in the north temperate region. These buds have been called winter-buds, resting buds, hibernacula and turions.

These specialised buds are similar to the dormant buds of terrestrial plants in that they serve as a means of surviving periods of unfavourable conditions for growth. However, unlike most buds of terrestrial plants, turions also act as propagules and in some species may be the major means of reproduction (Weber, 1972).

The Lemnaceae are no exception. Some species can adapt to adverse environmental conditions by producing dormant or resting fronds (turions), which abscise and sink to the bottom of the pond, lying dormant until conditions are more favourable for growth. The most studied turion is that of Spirodela polyrrhiza. They are smaller and thicker than vegetative fronds and are kidney-shaped (Jacobs, 1947; Henssen, 1954). The turion is about two-thirds as broad as long and is usually about 2 mm in width; while the vegetative fronds are usually 5 mm long and 4 mm wide, and often reach a length of 8 mm. The pigmentation of the turion is known to depend on environmental factors, with those formed from vigorous mother fronds at high temperatures and light intensities being dark green above, and reddish below. The turion is dull while dormant, but becomes glossy on 'germination' (Jacobs, 1947).

The stolons which are attached to the turions are much shorter than those which bear vegetative fronds (Jacobs, 1947), and the abscission layer at the proximal end of the turion is very well developed (Newton et al., 1978). Because of this the turion abscises readily and sinks when fully mature. The vegetative frond has 2 abscission layers, the first to mature being the one between the daughter and its stolon from the mother. The stolon later abscises from the daughter frond (Witzum, 1974b). Adjacent to the single

abscission layer in the turion are darkly staining cells, referred to as the protective layer by Newton et al. (1978), and these cells remain with the turion as a dark ring at the proximal end.

According to Jacobs (1947), the epidermal cells of the turion are similar to those of the vegetative frond but the stomata appear closed until after germination. The parenchymatous cells inside are filled with starch grains and are compactly arranged with only small intercellular spaces (Hegelmaier, 1868; Guppy, 1895; Jacobs, 1947). Subepidermal idioblasts (darkly staining cells) were reported to be concentrated on the dorsal and ventral sides of the turion, and the cells of the turion are generally heavily pigmented with anthocyanin. These idioblasts contain flavin-like compounds (Witzum, 1974a) and may have a protective function (Esau, 1965). The proximal upper surface is covered with a pocket sheath also pigmented with anthocyanin (Newton et al., 1978).

Several primordial fronds are contained in the pockets of the dormant turion of S. polyrrhiza. The largest and first to develop is less than 0·4 mm in length. A small axillary bud is usually present but this rarely develops, as the turion usually dies after the first frond has emerged. The other pocket contains the second primordium (which may develop) and very rarely the fourth. Turions always develop vegetative fronds and never produce turions directly (Jacobs, 1947). The dormant turion has no roots but may have root primordia embedded in the tissue at the node. These may elongate during germination and push through the epidermis.

While turions have been observed in many members of the Lemnaceae, none are as obviously modified as those of Spirodela

polyrrhiza (Thompson, 1898; Hicks, 1937; Landolt, 1957). Landolt (1957) reported turion formation in clones of Lemna minor and 3 species of Wolffia. Wolffia turions are similar to vegetative fronds except that they contain large amounts of starch (Hegelmaier, 1868; Landolt, 1957; Godziemba-Czyz, 1969a, 1969b). Turion formation has been reported in Wolffiella floridana (Pieterse et al., 1970) and in a new species, Lemna turionifera (Landolt, 1975). Turions of Wolffiella floridana, like those of S. polyrrhiza, sink to the bottom of the supporting medium and structurally are composed of small starch filled cells, and could also be induced by sucrose (cf. Henssen, 1954, Czopek, 1963). Unlike S. polyrrhiza turions those of Wolffiella regained normal vegetative growth when transferred to normal medium, and could also produce daughter turions. They generally germinated while submerged. On reflection these 'turions' bear more similarity to the submerged fronds of Wolffia arrhiza, which as Godziemba-Czyz pointed out (1969a) are quite distinct from the turions.

1.9 TURION FORMATION IN THE LEMNACEAE

Turions of Spirodela polyrrhiza were first described by Hoffman in 1840, although field studies on the turions of this species were not recorded until later (Hegelmaier, 1868; van Horen, 1870; Guppy, 1895). Van Horen (1870) also reported the formation of less modified turions in Lemna gibba. Detailed studies on turion formation in S. polyrrhiza did not start until 1947 by Jacobs and later by Czopek (1959, 1963, 1967). Landolt (1957) apparently unaware of Jacobs' earlier work reported turion formation in

Spirodela polyrrhiza, Lemna minor and Wolffia spp., under controlled and aseptic conditions, but no details were given.

Jacobs (1947) studied the growth and formation of turions under varying conditions of temperature, light intensity and duration, but apparently not under aseptic conditions. He concluded that turions were formed under any condition that maintained photosynthetic levels above that of carbohydrate utilisation for growth and respiration e.g. increased CO_2 levels increased turion formation. His anatomical studies showed that turion primordia were indistinguishable from vegetative primordia until they were at least 0·4 mm long, but he concluded that their destiny had probably already been determined before they were 0·2 mm long.

In 1954 Henssen showed that S. polyrrhiza produced turions under various mineral deficiencies or by the addition of sugars. Sucrose induced turion formation under light and dark conditions, while glucose was only effective in the light. Although he monitored starch formation, amylase activity and pH changes, he was unable to find any causal relationship to turion formation. Lautner and Müller (1954) reported that red light was inductive in turion formation, although a later study by Malek and Oda (1979) showed no such effect.

Jacobs' conclusions have had support from more recent researchers using aseptic conditions. Czopek (1959) reported changes in the morphology of turions formed in the light and the dark and concluded that turion formation resulted from overcrowding on the surface of the medium. Exclusion of one or more inorganic

compounds from the medium was found to induce turion formation. Henssen's work with sucrose was confirmed by Czopek (1963).

Perry (1968) showed that turion formation in S. polyrrhiza could be induced by manipulations of light intensity, day length, temperature and concentration of nitrate in the medium. He tested different clones for their ability to form turions under these conditions, and found one clone from Puerto Rico which did not form turions under any of the conditions used.

Newton et al. (1978) have recently re-examined turion formation and have confirmed earlier work by Henssen (1954) and show that nitrate reduces the sucrose enhancement of turion formation (cf. Perry, 1968). The sucrose enhancement was shown to be a metabolic rather than osmotic effect. They also investigated the combined effects of nitrate, sucrose and potassium and calcium ions, and found that calcium stimulated turion production when increased nitrate and sucrose were present, and suggested that calcium was enhancing sucrose uptake by increasing membrane integrity. Covey (1972) additionally showed that turion formation in the absence of sucrose was induced by depletion of nitrate or phosphate from the medium, but not by omission of calcium or magnesium.

Czopek (1967) also followed the changes in rates of respiration and photosynthesis during four phases of turion morphogenesis. Using Warburg manometric techniques and infra-red gas analysis, it was shown that the photosynthetic rate of newly formed turions was slightly higher than the respiratory rate. After one month in the dormant state the respiratory rate had increased and the photosynthetic rate decreased. Maximum rates of photosynthesis and respiration were

attained soon after turion germination and these rates gradually diminished as the turion died. While dormant, the rate of photosynthesis in the turion was found to be 25% of that of the vegetative frond.

The colouration of the turion is due to the presence of chlorophyll, carotenoids, anthocyanins and flavonoids. Reznik and Menschick (1969) have characterised a number of anthocyanins and flavonoids in both vegetative fronds and turions of S. polyrrhiza. From the turions these workers isolated one additional anthocyanin and eight additional flavonoids compared to the vegetative fronds. These extra components typically showed a higher degree of glycosylation. It is apparent therefore that the onset of turion formation somehow enhances secondary metabolism to synthesise these products.

1.10 TURION GERMINATION IN THE LEMNACEAE

Prior to germination of the turion of S. polyrrhiza, a bubble of gas forms from under the pocket (Newton et al., 1978; Jacobs, 1947), a young frond grows from one of the pockets, the roots of the turion begin to elongate and the turion rises to the surface. Germination occurs rapidly after storage for 2 weeks at 10°C and subsequent exposure to light (Henssen, 1954). The dormancy of naturally occurring turions can be broken by potassium cyanide in the light and dark, but only in the light with sugar induced turions. Czopek (1959) reported turion germination in the dark on inorganic media, but obtained a low germination rate. Germination in response to light has been attributed to the phytochrome system (Czopek, 1962,

1964). Kinetin or red light stimulated dark germination, while far-red light would reverse this effect. The effect of kinetin was also found by Lacor (1969), where it stimulated both light and dark germination.

A vernalisation requirement for turion germination has been reported for turions induced by manipulations of temperature, photoperiod and light intensity (Jacobs, 1947; Perry, 1968) and those induced by sucrose (Czopek, 1959; Newton et al., 1978). However, Sibaski and Oda (1979) reported that S. polyrrhiza turions formed in response to nitrogen deficiency germinated readily without pre-treatment. The germination of S. polyrrhiza turions was found to be inhibited in the dark (Czopek, 1964) and stimulated in the light (Lacor, 1969) by gibberellic acid.

1.11 IS THE TURION OF S. POLYRRHIZA A RESTING BUD?

The formation of turions in S. polyrrhiza shows many features in common with the development of resting buds in temperate woody plants. From a morphological viewpoint, the 'telescoping' of the bud scales and leaf primordia in the apical region, due to the arrest of normal internode development could be seen as analagous to the shortening of the stolon of the turion. The suppression of laminar development of the resting bud brings to mind the inhibited cell growth of the turion and lack of normal aerenchyma development. Both turion formation (Jacobs, 1947) and bud formation (Eagles and Wareing, 1964) have been shown to be under photoperiodic control.

Gibberellic acid is known to break the dormancy of a number of resting buds, including those with a chilling or photoperiodic

requirement. There is some evidence (Czopek, 1964; Lacor, 1969) that gibberellic acid can break the dormancy of turions in Spirodela polyrrhiza, and some turions are known to have a chilling requirement for germination. Kinetin has also been reported to overcome bud dormancy in woody plants (Wareing and Phillips, 1970), and Czopek (1964) reported the same in turions.

Furthermore, McLaren and Smith's work in 1976, showing that the utilisation of stored assimilate in the fronds of Lemna minor when they are returned to medium without ABA (cf. high starch and sugar levels in the turion), may be a phenomenon similar to bud burst in spring (van Overbeek and Mason, 1968).

1.12 TURION FORMATION AND ABSCISIC ACID

Perry and Byrne (1969) showed that abscisic acid concentrations as low as 4×10^{-8}M caused turion formation in Spirodela polyrrhiza in 10 days, and in 4 days with 4×10^{-7}M ABA. However, the clone from Puerto Rico (cf. Perry, 1968) did not form turions at any of the concentrations of ABA tested.

Stewart (1969) independently reported turion formation in S. polyrrhiza after 5 days in ABA (6×10^{-7}M). He made the observation that fronds formed from primordia initiated before the addition of ABA were intermediate in appearance between the vegetative frond and the turion, but commented no further. He also studied the time course and reversibility of turion formation, by growing fronds at 3×10^{-6}M ABA and transferring samples at intervals to medium free of ABA. 24 hours in ABA was found to be sufficient to initiate the biochemical events leading to turion

formation, the sequence of events being readily reversible. He speculated that a series of sequential events were responsible for turion formation, and that turion formation was not a result of growth inhibition alone (cf. Czopek, 1963). Kinetin was found to antagonise the turion inductive effect of ABA with a concomitant increase in the amount of growth inhibition. Kinetin also suppressed growth and turion formation in cultures with naturally occurring turions, which suggested to Stewart that ABA was indeed the endogenous factor controlling turion formation in natural conditions. Unfortunately, the criteria used by Stewart to detect turion formation were not reported.

Van Staden and Bornman (1969), studying the effects of varying concentrations of ABA in S. oligorrhiza, found that ABA halted growth at concentrations as low as 4×10^{-8}M, but no turions were observed. Indeed turion formation has never been reported in this species. Growth inhibition by ABA was also observed without turion production in Lemna minor (van Overbeek et al., 1967; van Overbeek and Mason, 1968). The fronds were "nearly completely dormant" at 4×10^{-6}M ABA, but could be revived by transfer to medium containing no ABA or by the addition of benzyladenine. It is notable that reversal of growth inhibition by benzyladenine could only be achieved at low concentrations of ABA. ABA was shown to inhibit nucleic acid synthesis with inhibition of DNA synthesis preceding that of RNA synthesis. This inhibition was reversed by benzyladenine which led these authors to conclude that ABA and cytokinins were allosteric inhibitors and activators respectively of DNA polymerase.

The hypothesis that ABA causes turion formation through its

effects as a general inhibitor of DNA synthesis appears to be untenable. Stewart and Smith (1972) working with S. polyrrhiza, showed that in the presence of ABA the incorporation of tritiated thymidine into DNA, separated by polyacrylamide gel electrophoresis, is inhibited by 80 - 90% in 3 hours. The incorporation of radioactivity into RNA was not inhibited until 3 - 9 hours, but reached an inhibition of 70% after 24 hours. There was little inhibition of protein synthesis until 2 - 3 days after ABA application. However, inhibitors of DNA, RNA and protein synthesis at none of the concentrations tested led to the formation of turions. It should be noted that these authors used ABA at a completely growth inhibiting concentration (i.e. 10^{-5}M); and did not mention whether at any time during their experimentation, turions were formed. The relevance of the observed inhibition of nucleic acid synthesis to turion formation is therefore not clear.

The most conclusive evidence to date that ABA is the endogenous promotor of turion formation in S. polyrrhiza comes from the work of Saks et al. (1980) who showed that the medium in which a turion forming culture (induced by a short photoperiod) had been growing, inhibited growth and induced turions in a fresh culture (grown under a long photoperiod), and that the released factor was ABA as shown by thin layer and gas chromatography. Moreover, the time of appearance of ABA in the medium and the rise in concentration during the following days correlated with the onset and extent of turion formation. The situation is less clear for turion formation in other aquatic plants. ABA has been shown to induce turion formation in Utricularia vulgaris under non-environmentally inductive

conditions (Winston and Gorham, 1979), enhance turion formation in Myriophyllum verticillatum under marginally inductive conditions (Weber and Nooden, 1976) and to have no effect in Ceratophyllum demersum (Best, 1979).

1.13 ABSCISIC ACID INDUCED BIOCHEMICAL MODIFICATIONS

Since its isolation in 1965, a considerable amount of information has accumulated about various aspects of ABA, a few of which, regarded as possibly relevant to the mechanism of dormancy, are discussed below.

1.13.1 Regulation of Replication

The first observations of the effects of ABA on nucleic acids were made by van Overbeek et al. (1967) using Lemna minor. They showed that ABA inhibited the incorporation of ^{32}P into all nucleic acid fractions and that benzyladenine reversed the process. The authors concluded that control of DNA synthesis was probably the prime effect of ABA, and speculated that DNA polymerase was an allosteric enzyme and that ABA and cytokinins could act as antagonistic allosteric effectors. The observed inhibition of RNA synthesis was thought to be a consequence of the diminuition in DNA synthesis.

While there is good evidence (Stewart and Smith, 1972; Sarrouy-Balat et al., 1973) that ABA does inhibit DNA synthesis in dividing tissues, other work has indicated that DNA synthesis is almost certainly not the primary target of ABA (Villiers, 1968; Haber et al., 1969; Chen and osborne, 1970; Walton et al., 1970), and that any inhibition of DNA synthesis by ABA is probably due to a

preceding effect on cell division.

1.13.2 Regulation of Transcription of Isolated DNA and Chromatin

There have been few studies of the effects of ABA on cell-free transcription. It was demonstrated that ABA treatment of non-dormant pear embryos reduced the transcriptional capacity of isolated chromatin and caused an inhibition of the increase in transcriptional capacity resulting from kinetin treatment (Khan, 1972). There is indirect evidence however, that ABA acts on chromatin in conjunction with a cytoplasmic factor, since adding ABA directly to purified chromatin of radish hypocotyl tissue had no effect on transcription. Transcription was however inhibited if ABA was added to the tissue homogenising buffer. Paradoxically pre-treatment of the hypocotyls with ABA did not quantitatively effect their transcriptional capacity (Pearson and Wareing, 1969). Although it is clear that ABA can reduce the capacity of chromatin to direct RNA synthesis, neither of these studies indicated whether polymerase activity or template availability had decreased.

1.13.3 Regulation of Polymerase Activity

Bex (1972a, 1972b) found that ABA inhibited RNA synthesis in maize coleoptiles and this was associated with a reduction in the activity of a partially purified soluble RNA polymerase not bound to chromatin. *In vitro* application of ABA to the grinding medium or to a polymerase-calf thymus DNA system resulted in inhibition of polymerase activity, indicating that ABA acted independently of the template by modulating polymerase activity. However, ABA inhibited *in vivo* RNA synthesis before any effect on polymerase activity could

be detected (Bex, 1972b).

1.13.4 Regulation of RNA Metabolsim In Vivo

Studies on radish leaf discs (Wareing et al., 1968), barley leaf segments (Poulson and Beevers, 1970), maize coleoptiles (Bex, 1972a), Avena coleoptiles (Cline and Rehm, 1974), embryonic bean axes (Walton et al., 1970), germinating pear embryos (Khan and Heit, 1969), Phaseolus vulgaris seedlings (Walbot et al., 1975), Lemna (van Overbeek et al., 1967) and Spirodela (Stewart and Smith, 1972) indicate that ABA does inhibit the synthesis of all RNA species.

However, there is increasing evidence suggesting that ABA inhibits growth by affecting the synthesis of minor RNA species (Paranjothy and Wareing, 1971; Walton et al., 1970; Villiers, 1968), and the overall inhibition of RNA synthesis may be a result of such growth inhibition. Indeed inhibition of growth often precedes any inhibition of RNA synthesis (Newton, 1974, 1977). Moreover, the available evidence suggests that ABA does not exert a general inhibition of transcription, but may inhibit the synthesis of specific species of mRNA. Thus in the barley aleurone ABA has little effect on total incorporation of labelled precursors into RNA, although it does inhibit GA-induced labelling of poly(A) RNA (Ho and Varner, 1974). Moreover, in this tissue ABA inhibits α-amylase synthesis and studies involving in vitro translation of α-amylase mRNA show that the inhibition of GA-induced α-amylase synthesis is associated with a reduction in the level of α-amylase mRNA (Higgins et al., 1977).

Further work has suggested that ABA inhibits α-amylase

synthesis by inhibiting the accumulation of translatable mRNA (Jones and Jacobsen, 1978; Jacobsen et al., 1980). On the other hand, Ho and Varner (1976) suggested that the inhibition of α-amylase synthesis by ABA is due to an effect on translation, since ABA still inhibited the formation of α-amylase at 12 hours, a time when the RNA synthesis inhibitor cordycepin no longer did. If ABA and cordycepin were added together at 12 hours, the inhibitory effects of ABA were eliminated. These authors therefore suggested that ABA might act by de-repressing a regulator gene or by interacting with a regulatory RNA or protein species to inhibit the translation of α-amylase mRNA.

Mozer (1980) has recently compared the patterns of protein synthesis in barley aleurone layers treated with GA_3 and ABA, with the patterns observed in in vitro cell free translation assays directed by RNA isolated from similarly treated layers. He concludes that while both hormones probably induce changes in both the transcription of mRNAs and the translation of mRNAs in barley aleurone, ABA does not interfere with GA_3 induction of translatable mRNAs, but prevents their translation in vivo.

In addition to the inhibitory effects of ABA in the barley aleurone system, ABA treatment causes the appearance of several new polypeptides and mRNAs (Ho, 1979; Jacobsen et al., 1980; Mozer, 1980). These proteins are formed in the absence or presence of GA_3, have short half-lives and are sensitive to both translation and transcription inhibitors.

There is also good evidence from other systems that ABA inhibits protein synthesis not through effects on mRNA transcription

but by affecting some later stage such as mRNA processing, translatability or stability. Ihle and Dure (1970) working with cotton embryos produced evidence that *in vivo*, ABA prevented the translation of mRNA in the late stages of embryogenesis. This study relies on the premise that during germination, the appearance of certain enzyme activities was not affected by actinomycin D and hence was apparently dependent upon preformed mRNA synthesis during embryogenesis. Reports that this inhibitor may have been ineffective because of poor uptake have raised questions about the interpretation of Dure's results (Smith *et al.*, 1974; Radin and Trelease, 1976). These authors provided evidence that isocitrate lyase was not translated from preformed mRNA, which is more in keeping with this enzyme's development in other fatty seeds.

Gayler and Glasziou (1969) also obtained evidence of a non-transcriptional control by ABA in studies on sugar cane discs, where ABA stimulated invertase synthesis. Similarly inhibitors of transcription were ineffective over the same time period that ABA was effective. Since these authors found no post-translational effect of ABA on invertase synthesis, they concluded that the site of action of ABA was at some post-transcriptional point. Similarly the enhancement of PEP-carboxylase and NADP-specific malic enzyme by ABA in *Lemna minor*, was shown to be unaffected by actinomycin D, but severely inhibited by cyclohexamide (Sankhla and Huber, 1979).

ABA may also control the stability and lifetime of RNA. Associated with reduced levels of RNA, ABA often causes an increase of RNAase levels as in barley leaf chromatin (Srivastava, 1968), in lentil roots (Pilet, 1970) and in *Avena* leaves (Wyen *et al.*, 1972).

Evidence against ABA exercising control over mRNA breakdown representing the main mode of ABA action, comes from the work of Bex (1972a) where there was no correlation between the rise in RNAase and the fall in RNA levels, and from Pearson and Wareing (1969) where ABA inhibited growth but had no effect on chromatin bound RNAase. Additionally, Trewavas (1970) found that while both the rates of synthesis and degradation of salt-soluble RNA (largely tRNA) of Lemna minor were reduced by ABA, the rate of degradation of salt-insoluble RNA (i.e. rRNA and mRNA) was unaffected.

Evidence for an effect of ABA not directly involving mRNA is scanty. Trewavas (1973) found that ABA inhibited both growth and the phosphorylation of a ribosomal protein in Lemna minor. Inhibition of growth followed the inhibition of phosphorylation and it was implicated that ABA induced changes in ribosomal protein phosphorylation may regulate ribosomal translational efficiency. The same author (1972b) also found that ABA could regulate protein levels by enhancing protein degradation.

1.13.5 Regulation of Photosynthesis and Related Metabolism

McLaren and Smith (1976) using Lemna minor, found that ABA inhibited the frond multiplication rate with an increase in dry matter production. By the use of other growth inhibitors they found that sugar and starch levels increased above that expected from growth inhibition alone, and indicated some specific effect of ABA. On transferral of ABA treated fronds to medium without ABA, a rate of growth higher than the controls resulted and the accumulated carbohydrates slowly decreased in quantity. ABA initially promoted CO_2 fixation for the first 2 days of the experiment, and caused a

gradual decline thereafter. McLaren and Smith (1976) concluded that the reduction in the photosynthetic rate after 10 days in ABA was a result of increased starch accumulation and subsequent reduced photochemical capacity.

ABA has also been shown to inhibit $^{14}CO_2$ fixation in Lemna minor by Bauer et al. (1976) and in a wide variety of other plants (Mittleheuser and van Steveninck, 1971; Poskuta et al., 1972; Sankhla and Huber, 1974, 1975; Maillard-Sevhonkian and Pilet, 1978). The short term enhancement of the photosynthetic rate found by McLaren and Smith (1976) has additionally been found with isolated protoplasts of Petunia (Hoffman and Kull, 1974).

There is good evidence that, while ABA may regulate photosynthesis by controlling the supply of CO_2 (unlikely in tissue with functionless stomata), it can also regulate photosynthetic enzyme activities, although there is no simple relationship between reduced rates of photosynthesis and enzyme acitivity. The activity of RuBP carboxylase was lowered in the leaves of cereals and Pennisetum (Wellburn et al., 1973; Sankhla and Huber, 1974, 1975) and apple seedlings (Ryc and Lewak, 1980) but RuBP carboxylase activity was increased by ABA in Lemna minor (Bauer et al., 1976; Sankhla and Huber, 1979). The activity of several other photosynthetic enzymes in Lemna minor was increased by ABA i.e. PEP carboxylase, NAD-malate dehydrogenase, NADP-specific malic enzyme, NADP-malate dehydrogenase and ribulokinase (Bauer et al., 1976; Sankhla and Huber, 1979); PEP carboxylase activity was increased in wheat leaves (Sankhla and Huber, 1975) and apple seedlings (Ryc and Lewak, 1980).

Bauer et al. (1976) have additionally shown that ABA inhibited

the Hill activity of isolated chloroplasts of L. minor by 50% and concluded that ABA might have a direct effect on the electron flow of photosystem II. The suggestion that ABA may be able to regulate the distribution of photosynthetically assimilated carbon in Pennisetum (Sankhla and Huber, 1974) was discounted by Bauer et al. (1976) and McLaren and Smith (1976) who found that ABA had no such effect in L. minor.

ABA has been shown to affect plastid development in wheat (Wellburn et al., 1973) and the ultrastructure of the chloroplast in L. minor (McLaren and Smith, 1976). McLaren and Smith (1976) also found that ABA increased the ratio of chlorophyll a/b, carotenoid levels and the starch grain size.

There is no evidence to date however, that photosynthesis or related carbohydrate metabolism are directly regulated by endogenous ABA.

1.13.6 Regulation of Other Aspects of Cellular Metabolism

While most ABA research has concentrated on aspects of transcription and translation, and to a lesser extent on photosynthesis, there is a small body of evidence suggesting that ABA is involved with the regulation of a number of other processes e.g. anthocyanin synthesis, amino acid metabolism and respiration.

While there is little information about the effects of ABA on anthocyanin synthesis, ABA has been shown to inhibit anthocyanin synthesis in radish (Guruprasad and Laloraya, 1980), but to have no effect on sunflower cotyledons (Servattaz et al., 1975). The activity of phenylalanine ammonia lyase, an enzyme which may be important in anthocyanin biosynthesis, is promoted by ABA (Walton

and Sondheimer, 1968).

Andres and Smith (1976) showed that ABA causes an increase in the free amino acid content of the fronds of Lemna minor, Lemna paucicostata and Spirodela polyrrhiza within 6 - 12 hours. Before this time a substantial increase in amino acid efflux into the medium was detected. These authors point out that while efflux into the medium may indicate an effect of ABA on cell membrane permeability, the increase in amino acid content inside the tissue could be due either to increased synthesis or decreased degradation. Increased synthesis seems unlikely in view of the findings of Bauer et al. (1976) and McLaren and Smith (1977) that the incorporation of $^{14}CO_2$ into amino acids was decreased significantly by ABA. ABA is also reported to increase the amino acid content in Phaseolus vulgaris (Huber et al., 1977).

There are also conflicting reports on the effect of ABA on respiration. Despite the effect of ABA upon α-amylase activity and starch hydrolysis in barley endosperm tissue, respiration was unaffected (Chrispeels and Varner, 1966). ABA treatment of Lemna minor which resulted in increased carbohydrate accumulation, caused an inhibition of respiration (McLaren and Smith, 1976), while ABA stimulated the onset of respiration in senescing Rumex leaf discs (Goldthwaite, 1974), and promoted oxygen uptake in potato pith discs (Hemberg, 1978). ABA had no effect on respiration during the early germination of lettuce seeds (Bex, 1972c) and inhibited respiration only marginally in germinating bean axes (Walbot et al., 1975).

1.13.7 Regulation of Ion Transport by ABA

One of the most extensively studied aspects of ABA is its ability to regulate stomatal aperture. Although there is a large body of literature on this aspect of regulation by ABA, it is not discussed in detail, since its relevance to the role of ABA in dormancy is unclear.

Wright and Hiron (1969) observed that placing leaves under water-stress induced a 40-fold increase in ABA concentration. It has since been postulated that water-stress results in an increase of ABA which induces stomatal closure and this leads to a decrease in transpiration and photosynthesis (Cummins et al., 1971). It was shown that exogenous ABA caused stomatal closure (Jones and Mansfield, 1970), inhibited stomatal opening (Horton, 1971) and decreased the photosynthetic rate within a few minutes of increased stomatal resistance (Mittleheuser and van Steveninck, 1971). The action of ABA is very rapid, but the time as well as the time to complete closure can vary depending on the type of plant and the amount of endogenous ABA present (Kriedemann et al., 1972).

Mansfield and Jones (1971) and Horton and Moran (1972) using histochemical techniques have shown that ABA inhibits the influx of K^+ into guard cells. In ABA induced stomatal closure, the osmotic pressure of the guard cells fall, the level of K^+ decreases and the amount of starch in the chloroplasts increases. These authors suggested that organic acids could provide the counterion for K^+. Support for this suggestion has come from the observation that ABA increased the extent of malate leakage from epidermal strips (Dittrich and Raschke, 1977; van Kirk and Raschke, 1978).

On the other hand, an exchange of H^+ has been proposed because stomatal opening can be induced by fusicoccin (Raschke, 1975). Moreover, fusicoccin overcomes the effect of ABA on stomata, apparently by stimulating the H^+/K^+ exchange system (see Marrè, 1979).

The conclusion that the primary effect of ABA on stomata could be upon proton expulsion from the guard cells (Raschke, 1975) is not entirely satisfactory since fusicoccin does not completely reverse ABA induced stomatal closure (Tucker and Mansfield, 1971).

Apart from the possible role of ABA in regulating K^+ exchange in stomatal guard cells, effects of ABA on K^+ uptake have been demonstrated in other plant responses. There are however, conflicting reports about the effect of ABA on ion uptake and accumulation.

Reed and Bonner (1974) found a selective inhibitory effect of ABA on K^+ uptake in elongating Avena coleoptiles, and a slight inhibition of Cl^- uptake. Proline and 3-O-methyl-glucose uptake was unaffected, and they suggested that ABA might act on specific ion carriers in the membrane rather than as the result of a general change in membrane permeability. Other evidence that ABA may act on cell membranes was provided by Shaner et al. (1975) who observed an inhibition of uptake and accumulation of K^+ (as ^{86}Rb) in 'low salt' roots of maize and a partial depolarisation of the membrane potential with ABA. They concluded that ABA had no effect on the passive diffusion potential and therefore must affect an electrogenic pump. However, they were unable to show any changes in the Mg^{2+} or K^+ stimulated ATPase, although a membrane bound Mg^{2+} activated

ATPase from tobacco leaves has been shown to be stimulated by ABA, thus resulting in increased proton influx (Kasamo, 1979).

On the other hand, ABA stimulated K^+ accumulation in 'low salt' roots of barley (Behl and Jeschke, 1979) and also caused an increase in the accumulation and uptake of Na^+ into the vacuole, although transport of both ions to the xylem was inhibited by ABA. Cram and Pitman (1972) using 'high-salt' roots report that ABA does not inhibit uptake but that it inhibits both water flow and net K^+ transport leading to an accumulation of K^+, the reduction of water flow being completely accounted for by the osmotic consequence of the K^+ accumulation. These authors showed that ABA addition could however lead to either a stimulation or inhibition of ion transport depending on conditions under which the plants were grown and on the experimental temperature.

ABA has been found to inhibit the rate of K^+ uptake and transport in sunflower roots (Erlandsson et al., 1978) and in Phaseolus vulgaris seedlings (Karmoker and van Steveninck, 1979). Both Cl^- and Na^+ uptake was stimulated however, although ABA inhibited the long distance transport of these ions from the root to the shoot (Karmoker and van Steveninck, 1979). K^+ uptake was inhibited in Vicia faba leaf slices (Horton and Bruce, 1972), while ABA delayed the initial development of the uptake capacity for K^+, Na^+ and Cl^- (van Steveninck, 1972) in beetroot discs, but later caused a large stimulation of their uptake.

ABA has also been implicated in the control of water movement other than that brought about through stomatal closure. ABA has been shown to affect both hydraulic conductivity and diffusional

permeability of water in root and pith cells (Glinka and Reinhold, 1971), while the action of ABA on diffusional permeability only, appeared to require metabolic energy (Glinka and Reinhold, 1972). ABA promoted root exudation through an enhanced hydraulic conductivity (Glinka, 1973).

1.14. AIMS OF THIS PROJECT

The search for correlations between the levels of ABA and the development of tree bud dormancy has led to no clear-cut support for any working hypothesis. Attempts to establish correlations have not been entirely unproductive, since the idea of a dormancy inducing substance led directly to the discovery of ABA, although the precise role of this growth substance in the control of vegetative growth remains to be elucidated. On the other hand any further efforts to test various hypotheses by measuring gross changes in the concentration of ABA in plant extracts may only confuse the issue further by producing yet more conflicting data. Continued emphasis on this approach diverts attention from the need to build up a comprehensive body of information on the precise nature of the cytological, biochemical and molecular events accompanying various stages in the development of dormant buds.

As far as acquiring information about the potential effects of endogenous growth regulators is concerned, it would seem more fruitful to study the effects of applied growth regulators in systems where the experimental variables are more readily controlled and where rather more specific changes than bud set or bud burst are measured. Some progress in this direction has been achieved by

Altman and Goren (1974) who have succeeded in maintaining buds of Citrus in culture on a defined medium.

The turion of Spirodela polyrrhiza was chosen for this project since it is inducible by ABA, can easily be manipulated at all operational stages without fear of microbial contamination, and thus represents a unique system in which to study aspects of development and its regulation by ABA in some detail. As is evident from a survey of the current literature, information regarding the ultrastructural and biochemical changes inherent in turion formation is virtually non-existent. This project was aimed at remedying these serious gaps in our knowledge of turion formation, using a combined ultrastructural and molecular approach, in order to understand better the mechanism of dormancy.

The approach manifest in this work is in essence therefore tri-tiered, involving initially a) characterisation of the morphological changes and structure of the vegetative frond and turion at both the light and electron microscope level, and investigating the cellular differences between frond and turion cells by detailed stereological analysis. In parallel b) a comprehensive examination and evaluation of some of the more accessible biochemical parameters associated with the mother frond and the subsequent turion and c) on a more fundamental molecular level, the relationship and possible consequences of the observed changes/differences in the mRNA and polypeptide profiles of the developing turion. Additional studies embraced those aspects of ABA induced action that effected responses primarily at the membrane level.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1 Chemicals

A23187 ionophore (H)

Abscisic acid, cis-trans isomer (B)

Acetaldehyde, reagent grade (A)

Acetic acid, glacial, analar (A)

Acetone, analar (A)

Acrylamide, specially purified for electrophoresis (A)

Adenosine 5'-triphosphate, disodium salt from equine muscle (B)

Adenosine 5'-triphosphate, vials (B)

Agarose, low EEO (B)

Albumin, bovine, fraction V powder (B)

Aldolase, type X from rabbit muscle (B)

Amido black 10B, grade II (B)

Amino acids, kit of 21 L-amino acids (B)

4-Aminosalicylic acid, sodium salt, reagent grade (A)

Ammonium persulphate, analar (A)

Ammonium nitrate, analar (A)

Ampholines, pH 3.5 - 10 (J)

Araldite cy212 (C)

L-Azetidine-2-carboxylic acid (B)

Boric acid, analar (A)

Brilliant blue G, purified (B)

Brilliant blue R (B)

Bromophenol blue, reagent grade (A)

Butyl-PBD (A)

Calcium nitrate, hydrated, analar (A)

Carbonic anhydrase, from bovine erythrocytes (B)

Casein, acid hydrosylate, type I (B)

Catalase, purified powder from bovine liver (B)

Charcoal, decolourising powder, activated, acid washed (A)

Chloroform, analar (A)

Cobaltous chloride, analar (A)

Cobaltous nitrate, analar (A)

Creatine phosphate, disodium salt (K)

Creatine kinase, from rabbit muscle (K)

m-Cresol, reagent grade (A)

Cupric sulphate, analar (A)

DDSA hardener (C)

Deoxyribonucleic acid, sodium salt, type I from calf thymus (B)

7X tissue culture detergent (L)

o-Dianisidine dihydrochloride, vial (B)

Diethyl ether, analar (A)

Dimethyldichlorosilane (B)

Diphenylamine, analar (A)

Dithiothreitol, reagent grade (A)

E-mix resin (C)

Ethanol, absolute, analar (E)

Ethanolamine, analar (A)

Ethidium bromide (B)

Ethylenediaminetetra-acetic acid (A)

Ethylenediaminetetra-acetic acid, disodium salt, analar (A)

Ethylenediaminetetra-acetic acid, ferric monosodium salt (A)

Ferrous sulphate, analar (A)

Firefly lantern extract, vial (B)

Folin & Ciocalteu's phenol reagent (A)

Formamide, analar (A)

β-D(-) Fructose (B)

D-Glucose, analar (A)

Glutaraldehyde, EM grade (C)

Glycerol, analar (A)

Glycine, analar (A)

Glycylglycine, free base (B)

Guanosine-5'-triphosphate, disodium salt (K)

Hydrochloric acid, analar (A)

Hydrochloric acid, aristar (A)

N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (A)

8-Hydroxyquinoline, analar (A)

Iodine, Jensen (A)

Instagel (N)

Invertase, grade X from yeast (B)

β-Lactoglobulin, from milk (B)

Lead nitrate, analar (A)

Lithium chloride, anhydrous (A)

Lysozyme, grade I (B)

Magnesium acetate, hydrated, analar (A)

Magnesium chloride, analar (A)

Magnesium sulphate, analar (A)

Manganous chloride, analar (A)

2-Mercaptoethanol, type I (B)

Methanol, analar (A)

NN'-Methylenebisacrylamide, specially purified for electrophoresis (A)

Myoglobin, from equine skeletal muscle (B)

Nonidet P40 (A)

Orthophosphoric acid, analar (A)

Osmium tetroxide, vial (C)

Perchloric acid, 72%, analar (A)

PGO enzymes, preweighed capsules (B)

Phenol, analar (A)

Potassium acetate (A)

Potassium chloride, analar (A)

Potassium dihydrogen orthophosphate, analar (A)

Potassium hydrogen carbonate, analar (A)

di-Potassium hydrogen orthophosphate, analar (A)

Potassium hydroxide, pellets, analar (A)

Potassium iodide, analar (A)

Potassium nitrate, analar (A)

Potassium sodium (+)-tartrate, analar (A)

Propylene oxide, EM grade (C)

Resorcinol, analar (A)

D-Ribulose 1,5-diphosphate carboxylase, from spinach (B)

Sephadex G25 (I)

Sodium acetate, anhydrous, analar (A)

Sodium cacodylate, EM grade (C)

Sodium carbonate, anhydrous, analar (A)

Sodium chloride, analar (A)

tri-Sodium citrate, analar (A)

Sodium dihydrogen orthophosphate, analar (A)

di-Sodium hydrogen orthophosphate dodecahydrate, analar (A)

Sodium hydroxide, pellets, analar (A)

Sodium hydroxide, 1M, carbonate free (C)

Sodium hypochlorite solution (E)

Sodium lauryl sulphate, specially purified for biochemical work (A)

Sodium molybdate, analar (A)

di-Sodium tetraborate, analar (A)

Spermidine, free base (B)

Sucrose, analar (A)

Sulphuric acid, analar (A)

NNN'N'-Tetramethylethylenediamine (B)

Toluene, analar (A)

Toluidine blue, for microscopical staining (A)

Trichloroacetic acid, analar (A)

Tri-iso-propylnaphthalenesulfonic acid, sodium salt, technical grade (G)

Triton X-100 (A)

Trizma, base, reagent grade (B)

Trypsin inhibitor, type 1S from soybean (B)

Uranyl acetate, analar (A)

Urea, analar (A)

Valinomycin (B)

Water, analar (A)

Wheat-germ (M)

Zinc sulphate, analar (A)

2.1.2 Photographic Materials

IF 23 (F)

Phen-X (F)

Microphen (F)

Pan F (F)

Kodachrome 64 (G)

Perceptol (F)

X-omatic H X-ray film (G)

Blue Brand Regulix (G)

2.1.3. Radiochemicals

Calcium-45, CES.3 (D)

Chlorine-36, CIS.1 (D)

L-(35S)Methionine, SJ204 (D)

Potassium-42 (D)

Sodium-22, SKS 1 (D)

(methyl-3H) Thymidine, TRK 418 (D)

(5,6-3H) Uridine, TRK 178 (D)

2.1.4 Scintillants

1. Butyl-PBD

Butyl-PBD	0·4 g
Toluene	100 ml

2. Triton/toluene

Butyl-PBD	0·6 g
Toluene	100 ml
Triton X-100	50 ml

3. Enhance (O)

4. Instagel (N)

2.1.5 Code to Suppliers

A BDH Chemicals Ltd.

B Sigma Chemical Co.

C EMscope Ltd.

D The Radiochemical Centre

E J. Burroughs Ltd.

F Ilford Ltd.

G Eastman Kodak Co.

H Lilly Laboratories

I Pharmacia Fine Chemicals

J LKB Instruments Ltd.

K Boehringer Mannheim Ltd.

L Flow Laboratories

M General Mills Co.

N Packard

O NEN Co.

2.2 METHODS (GROWTH, MICROSCOPY AND PHOTOGRAPHY)

2.2.1 Plant Material

The original clone of Spirodela polyrrhiza L. was obtained from the Union Canal, Edinburgh and was designated Spirodela polyrrhiza (UC). Fronds from this clone were sterilised by immersing briefly in ethanol, and then in 6% (v/v) sodium hypochlorite for varying periods of time (5 - 90 s). Fronds were then washed in three changes of sterile water and transferred to Hoagland's medium supplemented with 50 $mg.l^{-1}$ casein hydrosylate. This treatment kills the mother frond but any developing fronds within the mother pocket survive and are

sterile. The developing frond surviving the longest immersion time and showing no microbial contamination was used to start the new culture by transferring it to half-strength Hutner's medium (H/2). Another clone, designated Spirodela polyrrhiza (N) was obtained from Nottingham University.

2.2.2 Culture Conditions

Fronds of Spirodela polyrrhiza were grown under aseptic conditions on 100 ml of H/2 in 250 ml Erlenmeyer flasks, stoppered with a foam bung. The cultures were kept at a constant light intensity of 175 μeinsteins.$s^{-1}.m^{-2}$, produced from 9 warm white fluorescent tubes (40 W) and 2 tungsten tubes (60 W), at a temperature of 25 ± 1°C, and cultures were illuminated for 20 hours a day. Fronds were transferred to fresh medium using a sterile spatula in a stream of sterile air. Stocks were subcultured every 7 days to maintain a vigorous and non-senescing supply of material, and were checked for contamination by transferring fronds to sterile nutrient broth solution supplemented with 2% (w/v) sucrose. Absence of cloudiness after incubation for one week at 37°C, was taken as evidence of sterility.

2.2.3 Growth Media

2.2.3.1 Hutner's medium

Composition

Compound	$mg.l^{-1}$	mM
K_2HPO_4	400	2·3
KOH	200	3·6
EDTA	500	1·7
NH_4NO_3	200	2·5

Compound	$mg \cdot l^{-1}$	mM
$Ca(NO_3)_2 \cdot 4H_2O$	354	1.5
$MgSO_4 \cdot 7H_2O$	500	2·0
$FeSO_4 \cdot 7H_2O$	24·9	0·09
$MnCl_2 \cdot 4H_2O$	17·9	0·09
$ZnSO_4 \cdot 7H_2O$	65·9	0·23
H_3BO_3	14·2	0·23
$CuSO_4 \cdot 5H_2O$	3·95	0·016
$Na_2MoO_4 \cdot 2H_2O$	25·2	0·10
$Co(NO_3)_2 \cdot 6H_2O$	0·2	0·0007

The following stock solutions were prepared:

1·A To 200 ml of water

$Ca(NO_3)_2 \cdot 4H_2O$	17·7 g
EDTA	25·0 g
K_2HPO_4	20·0 g
NH_4NO_3	10·0 g
KOH	12–15 g until solution is clear

B To 150 ml of water

$ZnSO_4 \cdot 7H_2O$	3·295 g
H_3BO_3	0·710 g
$Na_2MoO_4 \cdot 2H_2O$	1·260 g
$CuSO_4 \cdot 5H_2O$	0·197 g

add 6M HCl drop by drop until solution is clear

$Co(NO_3)_2 \cdot 6H_2O$	0·010 g
$MnCl_2 \cdot 4H_2O$	0·897 g

C To 50 ml of water

$FeSO_4 \cdot 7H_2O$ 1.245 g

Solutions A, B and C were mixed with constant stirring and made up to 1 litre with water.

2. $MgSO_4 \cdot 7H_2O$ 25.0 g

made up to 1 litre with water.

Stock solutions 1 and 2 were kept in sterile bottles at 4°C. 10 ml of stocks 1 and 2 were mixed for H/2 medium and made up to 1 litre with water, and the pH adjusted to 6.3 with 5M KOH. The medium was dispensed into the flasks, which were sealed with foam bungs, covered with aluminium foil and autoclaved at 15 p.s.i. for 15 min.

2.2.3.2 Hoagland's medium

Stock solutions were prepared as follows:

	Compound	$g.l^{-1}$
1.	Sucrose	342
2.	$Ca(NO_3)_2 \cdot 4H_2O$	118
	KNO_3	50
3.	$MgSO_4 \cdot 7H_2O$	49
4.	KH_2PO_4	27.2
5.	EDTA ferric monosodium complex	14.0
6.	H_3BO_3	2.86
	$MnCl_2 \cdot 4H_2O$	1.81
	$CuSO_4 \cdot 5H_2O$	0.08
	$ZnSO_4 \cdot 7H_2O$	0.22
	$Na_2MoO_4 \cdot 2H_2O$	0.10
	$CoCl_2 \cdot 6H_2O$	0.25
	KI	1 crystal

10 ml each of stocks 1-4 were mixed and made up to 500 ml with water. 1 ml each of stocks 5 and 6 were added and the solution made up to 1 litre. The medium was dispensed and autoclaved as for Hutner's medium.

2.2.4 Abscisic Acid

Crystalline ± cis-trans ABA was prepared as a 10^{-3}M stock solution by dissolving 13·22 mg in 1 ml of methanol, and diluting this to 50 ml with H/2, with magnetic stirring to avoid precipitation. This solution was then sterilised by millipore filtration (0·22 μm), and all serial dilutions were performed using aseptic techniques. All ABA solutions were kept at 4^{o}C in the dark.

2.2.5 Ionophores and Inhibitors

1. Valinomycin

Crystalline valinomycin was prepared as a 10^{-3}M stock solution by dissolving 5 mg in 4·5 ml of acetone. This solution was further diluted with H/2 to 10^{-5}M before millipore filtration, and stored at 4^{o}C in the dark.

2. A23187

A23187 was prepared as a 10^{-3}M stock solution by dissolving 5·23 mg in 10 ml of ethanol. This was diluted to 10^{-5}M with H/2, filter sterilised before use, and stored at 4^{o}C in the dark.

3. Azetidine-2-carboxylic acid

Crystalline L-azetidine-2-carboxylic acid was prepared at 10^{-3}M by dissolving 5·05 mg in 50 ml of H/2, and filter sterilised before use.

2.2.6 Growth Determination

Frond multiplication rate was determined daily by counting the fronds in each flask. All fronds which visibly protruded beyond the margin of the mother frond were included in the count. In all experiments fronds were innoculated onto fresh medium and allowed to grow for one day, before ABA was added.

2.2.7 Dissection and Experimental Design

Many of the parameters studied in this work were determined not only on whole plantlets of S. polyrrhiza incubated in ABA, but also on dissected tissue. To monitor specific changes occurring in turion formation, the developing turions were dissected from the mother pockets and used separately for analysis. In some experiments plantlets were dissected into different frond size classes.

2.2.8 Fresh and Dry Weight Determination

Fresh weights were determined by pouring the fronds from the medium onto a Whatman no. 1 filter paper placed in a Buchner funnel attached to a vacuum line. The fronds were washed with water (2 x 100 ml), blotted on 2 layers of filter paper and weighed immediately. For dry weight determination the fronds were transferred to aluminium pastry cases and oven dried at 90^{o}C for 24 hours; longer drying periods resulted in no further weight decrease.

2.2.9 Preparation of Tissue for Microscopy

Segments of fronds were fixed in 3% (v/v) glutaraldehyde, 0·1 M sodium cacodylate buffer at pH 6·3 overnight at 4^{o}C. The tissue was rinsed with 6 changes of the cacodylate buffer for 3 hours, and then post-fixed in 1% (v/v) osmium tetroxide for 2 hours. The tissue was

then washed for 1 h with 3 changes of buffer and stored at 4°C overnight. The tissue was dehydrated in the following alcohol series:

25% (v/v)	ethanol	30 min
50%	" "	"
70%	" "	"
80%	" "	"
90%	" "	"
95%	" "	"
absolute	"	"
absolute	"	"
100% (v/v)	"	"
100% (v/v)	"	"

The tissue was rinsed in 4 changes of propylene oxide for 1 hour, an equal volume of epoxy resin was then added to the propylene oxide (araldite cy212/DDSA hardener) and the mixture stirred carefully to avoid disrupting the tissue. An equal volume of resin was added after 1 h, mixed and left overnight at room temperature. The resin was poured off, fresh resin added, and the mixture placed in a vacuum embedding oven for 1 h. The frond segments were then embedded in capsules for 24 h at 60°C. Later work was carried out using the low viscosity resin E-mix.

Thick sections (1 μm) of the embedded material were cut with glass knives on an LKB 4802A ultramicrotome, stretched on a hot-plate, stained with 1% (w/v) toluidine blue in 1% (w/v) borax, and examined with a Zeiss photomicroscope. Photographs were taken with Pan F film and developed in Perceptol for 17 min.

Ultrathin sections (70-95 nm) were also cut with glass knives, but were floated onto water, stretched with chloroform vapour and dried on copper grids (200 mesh). The sections were stained for 30 min in the dark with a saturated solution of uranyl acetate in 50% (v/v) ethanol, washed in 50% (v/v) ethanol and air dried. They were then stained in lead citrate (Reynolds, 1963) for 5 min, washed twice in carbonate free 0•02 M NaOH, then in water and finally air dried. Sections were examined with an AEI EM6 electron microscope.

Frond segments were embedded in the conical end of the capsule for paradermal sections of the tissue, which thus eliminated most of the coarse trimming of the block. Segments were embedded in the flat end of the capsule for longitudinal sections across the mother pocket. After embedding, the flat circular disc of resin containing the specimen was trimmed and orientated in a pincer type block holder, and serial thick sections were cut until the centre of the developing frond was located. This was either determined by taking the section containing the longest profile of the developing frond, or, if the tissue was orientated exactly, until the stolon connecting the young frond with its mother came into view.

2.2.10 Stereological Analysis of Frond and Turion Tissue

The approach chosen follows that of Weibel et al. (1969), which involves the preparation of a series of light and electron micrographs taken systematically of sections of the tissue in question. The micrographs were then sampled using a superimposed test system which allows the measurement of a range of parameters of the two-dimensional sections, such as the area fraction occupied by different cell or tissue components and the length of sectioned

interfaces (i.e. surfaces) per unit area. Such data, from a large number of sections, can be substituted into standard stereological formulae (see appendix) to provide information on the 3-dimensional structure of the tissue. These measurements can be used to calculate the volume fractions (volume density) and the surface areas per unit volume (surface density) respectively of tissue components. The application of these techniques to compare the vegetative frond with the fully developed turion permits an analysis of the differences in terms of cell and organelle size, and this in turn allows correlation of the structural differences with the major biochemical events occurring during turion formation.

2.2.10.1 Experimental protocol

A plantlet of five fronds was cut into small pieces, which were all fixed and embedded (without orientation). The same operation was performed on 5 fully developed turions. 10 blocks were selected randomly from each sample. It was upon this sample that subsequent light and electron microscopy was carried out.

The nature of the tissue required that structural analysis be carried out at a number of different magnifications to fulfil two conflicting requirements. These were, the need on one hand, to sample a sufficiently large area of the section, and on the other, to use a magnification high enough to resolve all the tissue structures. Consequently the sections were examined at a number of different magnifications using a Jeol JEM-100S electron microscope.

Level I

This was a low power study to determine the volume of tissue occupied by cells, air spaces, protoplasm, vacuoles and cell walls.

Point counts were made on toluidine blue stained 1 μm sections using a Weibel pattern 6 x 6·93 z test system, where z = 2 cm and the total number of points 48. One section was cut from each block and 5 photomicrographs were taken systematically from each section at a magnification of 160X. From this pool of 50 photomicrographs for each sample, 25 were randomly selected for enlargement to 1090X. The test system was placed on top of each micrograph and point counts were made on each. Volume densities of air spaces, cells, vacuoles, protoplasm and cell walls were estimated in $cm^3 . g^{-1}$ of tissue. The actual test area for each micrograph was $1·4 \times 10^{-4}$ cm^2.

Level II

While measurements made at level I were adequate to determine the major cell compartments, it was not possible to distinguish, for example, the many small vacuoles in the cytoplasm which can be seen under the electron microscope, or to resolve starch in the case of turion tissue. A low power EM survey was therefore used to determine the same parameters as for level I, and additionally the volume densities of starch and nuclei.

One section was cut from each block and 5 electron micrographs taken at a magnification of 1000X. Random sampling of the field was ensured by positioning the mark on the screen of the electron microscope exactly in the centre of 5 consecutive squares of the supporting copper grid. From this pool of 50 electron micrographs for each sample, 20 were randomly selected for enlargement to 2703X.

Two different test systems were applied to these micrographs, because of the rarity of certain components e.g. nucleus. A Weibel 6 x 6·06 z test system, where z = 2·5 cm and the point count was 42

was used to estimate volume densities of the vacuole, cell wall and protoplasm. The term 'protoplasm' is defined here as ground cytoplasm, organelles, starch and nuclei. The test area of each micrograph was $3{\cdot}11 \times 10^{-5}$ cm^2. A Weibel 14 x 12·99 z system, where z = 1 cm and total point count was 210, was used to resolve the vacuole, cytoplasm, starch, cell wall and nuclei. The lines of the test system were used to calculate the surface densities of the plasmalemma and the tonoplast. The test area of each micrograph was $2{\cdot}49 \times 10^{-5}$ cm^2.

Level III

At this level the volume composition of the cytoplasm in terms of its organelles was determined, and parameters were calculated in relation to the cytoplasmic volume (whose contribution to cell and tissue volume was determined at levels II and I respectively). 5 electronmicrographs were taken from each section at a magnification of 10,000X. Random sampling was ensured by positioning the sampling area in the centre and the 4 corners of one square of the grid. From this pool of 50 micrographs, 20 were randomly selected for enlargement to 27,030X. The 14 x 12·99 z, z = 1 cm test system was used, which gave a test area of $2{\cdot}49 \times 10^{-7} cm^2$. The volume densities of ground cytoplasm, chloroplast, mitochondria, endoplasmic reticulum, chloroplast stroma, thylakoids, and chloroplast envelope were estimated. Fields containing less than 50% cytoplasm were not recorded.

2.2.10.2 Analysis

Stereological calculations were carried out for each of the 2 samples (vegetative fronds and turions), for each parameter at each

level. Means and standard error of the means within each level were obtained as follows:

designating the parameter with x, and having studied x on representative micrographs one obtains

the mean $\bar{x} = \frac{\sum x}{n}$ where n is the number of micrographs, and

the standard error of the mean $SE = \sqrt{\frac{\sum x^2 - x\sum x}{n\,(n-1)}}$

The significance of the difference between samples was tested by the non-parametric Rank Sum Wilcoxon test for 2 samples (Documenta Geigy p. 192).

2.2.11 Photography

2.2.11.1 Macro-photography of turion development

Fronds with one or more daughters were innoculated in petri-dishes containing 15 ml of H/2 with or without ABA. The daily growth of individual fronds was monitored by photographing the dish from below, thus keeping the contents sterile. Pan F film was used rated at 100 ASA and developed for 7 min in Microphen to increase contrast. Photographs were enlarged to the same magnification and the lengths of the fronds calculated by comparison with a floating scale marker. This procedure was used to study both clones of Spirodela polyrrhiza. In preliminary experiments with S. polyrrhiza (UC) the growth of individual fronds was also followed using an inverted Prior microscope fitted with a long working distance objective. Drawings were made of each frond and its length measured with an eye-piece graticule.

In both types of experiment, 20 fronds were studied using different concentrations of ABA. It was possible to retrace the development of an individual frond back to day 0, by assigning each

frond a distinguishing letter. For any given exposure to ABA, any frond could then be characterised as a developing vegetative frond or a developing turion.

2.2.11.2 Colour photography

Colour photography was used in certain instances to show differences in colouration between the turion and the vegetative frond, and was particularly useful in demonstrating whether the process of turion formation could be reversed by removing ABA during the early stages (see section 3.2.3). Photographs were taken (with Kodachrome 64) using an Olympus PM6 camera fitted onto an Olympus Model X binocular stereoscopic microscope. Lighting was provided from a Natchet halogen lamp using two fibre optic probes pointing directly onto the specimen, through a filter to compensate for the daylight film. Transparancies were developed by Kodak and enprints were made by Max Spielmann Ltd..

2.2.11.3 Examination of the stomata of Spirodela polyrrhiza

Vegetative fronds were incubated either in H/2 alone or with the addition of ABA for either 1 hour or 10 days (which results in the formation of turions). Epidermal peels were made, stained with iodine and examined with a Zeiss photomicroscope. Stomata from the vegetative fronds and the turions were photographed, and the negatives developed at the Macaulay Institute, Aberdeen.

Fronds and turions were also frozen in liquid nitrogen and examined at $-120^{o}C$ in a Cambridge S150 scanning electron microscope fitted with a Series 200 heating and cooling specimen stage.

2.3 METHODS (CHEMICAL AND BIOCHEMICAL)

2.3.1 Chlorophyll Estimation

Fronds were homogenised in 50 mM Tris-Ac pH 8·5, 50 mM KAc, 5 mM $MgAc_2$, 2 mM DTT (grinding buffer), and 4 volumes of acetone added. Samples were left overnight at -20°C in the dark and centrifuged at 12,000 g_{max} for 5 min. The pellet was re-extracted with 80% (v/v) acetone until a colourless supernatant was obtained. The supernatants were pooled and the OD at 480 nm, 645 nm and 663 nm determined. Concentrations of chlorophylls a and b in the supernatants were calculated using the equation below (Arnon, 1949):

$$\text{chlorophyll a (mg. } l^{-1}) = 12{\cdot}7\ (OD_{663}) - 2{\cdot}69\ (OD_{645})$$

$$\text{chlorophyll b (mg. } l^{-1}) = 22{\cdot}9\ (OD_{645}) - 4{\cdot}68\ (OD_{663})$$

The concentration of carotenoids can be obtained from the following equations (McLaren, 1976):

$$C = OD_{480} + (0{\cdot}114 \times OD_{663}) - (0{\cdot}638 \times OD_{645})$$

$$\text{carotenoids (mg. } l^{-1}) = \frac{C \cdot 10}{2{\cdot}505}$$

Chlorophyll and carotenoid concentration per frond was determined using these equations and appropriate dilution factors.

2.3.2 Anthocyanin Estimation

Fronds were immersed in 2-3 ml of 0·1 M HCl, and allowed to stand in the dark for 3 h at room temperature. The resulting extract was filtered through Whatman no. 1 paper and the OD of the filtrate was measured at 510 nm. Anthocyanin concentration was expressed as pigment units per frond, where

$$\text{pigment units} = OD_{510} \cdot 50 \cdot \text{no. of ml of HCl}$$

Since 1 pigment unit was found to be equivalent to $6{\cdot}7 \times 10^{-4}$ mg of

cyanin (Thimann and Edmondson, 1949) and the anthocyanin of Spirodela polyrrhiza is cyanidin-3-monoglucoside (Krause and Strack, 1979), an approximate concentration can be calculated to give ng anthocyanin. frond^{-1}.

2.3.3 ATP Estimation

Fronds were homogenised in 2 ml of 0·5 M $HClO_4$ using a mortar and pestle (Obendorf and Marcus, 1974). The mortar was rinsed with 1·5 ml of water and the combined homogenates centrifuged at 23,000 g_{av} for 10 min, and the resultant pellet resuspended in 1 ml of 0·25 M $HClO_4$. Upon re-centrifugation, the supernatants were pooled and then neutralised with 1 M KOH containing 0·2 M $KHCO_3$ (~0·88 ml). The precipitate resultant after storage for 25 min on ice was centrifuged at 1,000 g_{av} for 10 min, and the supernatant stored at -40^{o}C. An internal standard was used to estimate the recovery of ATP during the extraction. This involved adding 50 µl of 10^{-4} M ATP to one extra sample of tissue before homogenisation.

ATP in the supernatants was estimated by the luciferin-luciferase assay (Humphreys, 1973). Light is emitted when luciferase splits ATP in the presence of luciferin. These flashes of light can be measured in a scintillation counter. The rate of light emission increases exponentially with the concentration of ATP. The emitted light decays rapidly after reaching a peak soon after the enzyme is introduced, although this peak is not usually encountered, since in practice more than 10 s elapses before the sample is introduced into the counter. To measure the ATP, sequential counts are taken from each sample.

Firefly lantern extract was reconstituted with 25 ml of water

and contained 0•05 M potassium arsenate and 0•02 M $MgSO_4$, pH 7•4. After dissolving overnight at 0°C, the suspension was centrifuged at 1,000 g_{av} for 5 min and the supernatant was dispensed into 2 ml aliquots and stored at -20°C. The assay was performed on an Intertechnique SL 30 scintillation spectrometer set out of coincidence, and with window settings optimal for ^3H. Glass scintillation vials (washed with 7X detergent, rinsed 10 times with hot water and 3 times with distilled water) were prepared, containing 0•9 ml of 0•04 M glycylglycine buffer, pH 7•4. Up to 50 µl of ATP standard (10^{-6} M) or unknown sample was added, followed by 0•1 ml of firefly lantern extract. The vial was swirled rapidly and put into the counter (without a cap) and counting started precisely 10 s after addition of firefly lantern extract. 6 consecutive 0•1 min counts were taken from each sample, and all samples were measured in duplicate.

In calculating the results, the counts from the 2nd 0•1 min period were used, and the ATP content in the unknowns calculated by reference to a standard curve from 0–50 pmole of ATP. Data were corrected for ATP lost during the extraction calculated from the internal standards.

2.3.4 Estimation of Sugars

Fronds were extracted 4 times with hot 80% (v/v) ethanol using a mortar and pestle. Following centrifugation at 1,000 g_{av} for 10 min, chlorophyll was removed from the supernatant by the addition of 1 ml of an activated charcoal suspension (135 g activated charcoal, 900 ml 50% (w/v) aqueous glycerol) to each 10 ml of extract. The supernatant was cleared by centrifugation and evaporated to dryness under vacuum. The residue was dissolved in 7 ml of water and the solution

used in the following assays.

2.3.4.1 Glucose 'PGO' enzyme assay

The procedure is based upon the following coupled enzymatic reactions, wherein glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. The hydrogen peroxide in turn, is acted upon quantitatively by the peroxidase which catalyses the transfer of the oxygen from the peroxide to a chromogenic oxygen acceptor, ortho-dianisidine:

a) $\text{glucose} \xrightarrow[+ H_2O + O_2]{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$

b) $H_2O_2 + \underset{\text{(colourless)}}{\text{ortho-dianisidine}} \xrightarrow{\text{peroxidase}} \underset{\text{(brown)}}{\text{oxidised o-dianisidine}}$

The intensity of the brown colour produced is a quantitative measure of the original glucose concentration.

1·5 ml of test solution (or 0–100 μg of β-D-glucose standard) was added to 0·1 ml of o-dianisidine dihydrochloride (2·5 mg. ml^{-1}) followed by 2·0 ml of 'PGO enzyme' preparation (Sigma Technical Bulletin no. 510). The samples were mixed, incubated for 35 min at 40°C and the OD read at 460 nm.

2.3.4.2 Sucrose

3·5 ml of test solution (or 0–250 μg of sucrose standard) was incubated for 30 min at 40°C with 2·0 ml of yeast invertase (20 μg. ml^{-1}) and 2·0 ml of citrate-phosphate buffer pH 4·5 (Documenta Geigy p. 281). The solution was then boiled for 5 min and a 1·5 ml sample taken for glucose assay (McLaren, 1976).

2.3.4.3 Fructose

Fructose was determined by a modification of Roe (1934). 1 ml of test solution (or 0–200 μg of β-D-fructose standard) was added to

0•5 ml of resorcinol (0•45% w/v) and 2•5 ml of copper reagent (8•4 mg $CuSO_4 \cdot 5H_2O$, 200 ml HCl, 260 ml glycerol, 100 ml water). The solution was then incubated at 100°C for 12 min and the OD read at 470 nm. Correction factors were applied as follows:

measured glucose = a

measured fructose = b

glucose after invertase = c, then

glucose = a

$$\text{sucrose} = \frac{342}{180}(c-a)$$

fructose = b − (c−a)

2.3.5 Protein Precipitation and Assay

2.3.5.1 TCA precipitation

Aliquots of total and soluble protein samples were precipitated with an equal volume of 10% (w/v) TCA and kept on ice for 30 min. The protein precipitate was pelleted by centrifugation at 12,000 g_{max} for 1 min, and washed twice in 5% (w/v) TCA. The final pellet was hydrolysed in 0•1 M NaOH overnight at 37°C.

2.3.5.2 Acetone precipitation

Aliquots of total and soluble protein samples were precipitated with 4 volumes of acetone and left overnight at -20°C. The protein precipitate was pelleted at 12,000 g_{max} for 1 min, and washed twice in 80% (v/v) acetone. The pellet was hydrolysed in 0•1 M NaOH overnight at 37°C.

For insoluble protein samples the pellets were washed twice with 50 mM Tris-Ac, pH 8•5, 50 mM KAc, 5 mM MgAc, 2 mM DTT (grinding buffer), and resuspended in the same buffer. An aliquot was taken

for acetone precipitation and the resulting pellet hydrolysed as before.

2.3.5.3 Lowry assay

The protein content of homogenate (total protein), 10,000 g_{av} supernatant (soluble protein) and the pellet (insoluble protein) samples was determined by an adaptation of the method described by Lowry et al. (1951), whereby production of a blue colour relies upon the reduction of the Folin reagent by the amino acids tyrosine, tryptophan and cysteine.

Aliquots of the protein hydrosylate were assayed in a final volume of 200 µl in 0·1 M NaOH, and used directly for protein estimation. The following solutions were prepared:

A. Fresh 2% (w/v) Na_2CO_3 in 0·1 M NaOH

B. 1% (w/v) $CuSO_4 \cdot 5H_2O$

C. 2% (w/v) K/Na tartrate

D. Folin and Ciocalteu's phenol reagent diluted 1:1 with water (1.2 N)

E. 50 ml of A + 0·5 ml of B + 0·5 ml of C.

A standard curve from 0-200 µg of BSA was prepared for each assay in a volume of 200 µl of 0·1 M NaOH. 1 ml of solution E was added to each 200 µl sample, mixed and allowed to stand for 10 min for the formation of the protein-copper complex. 100 µl of solution D was added to each sample and vortexed immediately. The OD was measured at 750 nm after 30 min. The protein content of the samples was determined relative to the BSA standard curve.

2.3.5.4 Bradford assay

This assay (Bradford, 1976) relies on the binding of a dye to

protein which results in a shift in the absorption maximum of the dye from 465 to 595 nm. This assay was used in addition to the Lowry assay, since substances such as amino acids, carbohydrates and SDS etc. do not interfere with the assay. The procedure followed that of Spector (1978).

Total and soluble protein samples were used directly without prior precipitation. Insoluble protein pellets were washed and resuspended in grinding buffer as described in section 2.3.5.2. Aliquots were either taken directly for assay, or were solubilised by re-centrifugation and incubation in 50 mM Tris-HCl pH 7·4, 5 mM $MgCl_2$, 5% (w/v) 2ME, 0·5% (w/v) SDS (SDS buffer) at 70°C for 30 min. An aliquot was taken for assay after clearing the sample by centrifugation. Standard curves from 0-100 µg of BSA were prepared for each assay in a volume of 100 µl of either grinding buffer or SDS buffer. The dye reagent was prepared as follows:
Brilliant blue G250 (100 mg) was dissolved in 50 ml of 95% (v/v) ethanol, and 100 ml of 85% (w/v) phosphoric acid added. The resulting solution was made up to 1 litre with water and filtered before use.

5 ml of dye reagent was added to each 100 µl of protein sample, mixed, allowed to stand for 5 min, and the OD was read at 595 nm. The protein content of the samples was determined relative to the appropriate standard curve.

2.3.6 Qualitative Extraction of Nucleic Acids

Nucleic acids were extracted at 4°C (Leaver, 1973) by the phenol-detergent procedure of Leaver and Ingle (1971).

Fronds were homogenised in 5 ml of extraction buffer (100 mM

Tris-HCl pH 8·5, 6% (w/v) PAS, 1% (w/v) TNS). The homogenate was mixed with an equal volume of phenol/m-cresol/8HQ (500 g/redistilled 50 ml/ 0·5 g; saturated with 100 mM Tris-HCl pH 8·5); then centrifuged at 1,400 g_{av} for 10 min at 4°C, and the aqueous phase further extracted with fresh phenol mix until the interface was clear. The upper phase was made 0·1 M with respect to NaAc pH 5·0 and the nucleic acids precipitated by the addition of 2·5 volumes of ethanol at -20°C overnight. The nucleic acid was recovered by centrifugation at 10,000 g_{av} for 10 min, washed twice with 80% (v/v) ethanol, 0·1 M NaAc pH 5·0 and dried under vacuum. The pellet was resuspended in water and high molecular weight RNA removed from solution by the addition of an equal volume of 6 M LiCl, and centrifugation at 12,000 g_{max} for 5 min, where only high molecular weight RNA was required. The RNA was re-precipitated with ethanol, washed as before, redissolved in water, and stored at -20°C.

The nucleic acid extracted by this method was used for electrophoretic fractionation and for programming a wheat-germ cell free protein synthesising system.

2.3.7 Determination of Inorganic Constituents

Known fresh weights of tissue were oven dried at 90°C and ashed at 550°C. The residue was taken up in 6 M HCl (aristar) and finally made up to 25 ml with 0·1 M HCl for chemical analysis. Iron was determined with 2,4,6-tripyridyl-s-triazine (Collins and Diehl, 1960) and phosphorus with molybdate and vanadate (Hanson, 1950).*

Calcium, magnesium, sodium and potassium were all determined by flame emission spectrophotometry (DeKock et al., 1975).*

* performed by Mr. A. Hall

2.4 METHODS (ELECTROPHORETIC)

2.4.1 Quantitative Estimation of RuBP Carboxylase Protein

Ribulose bis-phosphate carboxylase was determined by electrophoretic fractionation of soluble protein under non-denaturing conditions (Blenkinsop, 1973). The stained protein was recovered and quantitised after excision of the appropriate gel band.

Gels were cast to a length of 8 cm in perpex tubes (9 cm x 0·7 cm) sealed at the base with 2 layers of parafilm. The gel mixture was composed of 5% (w/v) acrylamide, 0·125% (w/v) bis-acrylamide, 87·5 mM Tris-glycine pH 9·5 and was polymerised with 0·033% (v/v) TEMED and 0·075% (w/v) AMPS. The gels were overlaid with water, allowed to set for 1 hour and pre-run for 30 min at 1 mA per gel with 50 mM Tris-glycine pH 9·5 electrode buffer.

Fronds were homogenised in 300 µl of grinding buffer (section 2.3.5.2) in a micro glass tissue grinder and a 50 µl aliquot taken for total protein determination (section 2.3.5). The remaining 250 µl of homogenate was centrifuged at 10,000 g_{av} for 10 min and volumes of the supernatant corresponding to 25 µg of total protein were loaded onto the gels in 50 mM Tris-glycine pH 9·5 containing 10% (w/v) sucrose and bromophenol blue as a tracker dye.

Electrophoresis was carried out at 1 mA per gel for twice the time taken for the dye to reach the bottom of the tubes. The gels were removed by gentle air pressure, stained in 0·1% (w/v) amido black 10B in 20% (v/v) ethanol, 7% (v/v) acetic acid, and destained in frequent changes of 20% (v/v) ethanol, 5% (v/v) acetic acid. Identification of the carboxylase band was achieved by co-electrophoresis with purified enzyme. The carboxylase band was

excised from the gel, placed in 2 ml of formamide, and incubated overnight at 50°C. The optical density of the resultant solution was read at 625 nm. Quantitation was achieved by preparing a standard curve for electrophoresis using spinach D-ribulose-1,5, bisphosphate carboxylase. Gel slices, containing ^{35}S methionine labelled protein from frond tissue, were dissolved in 1 ml of H_2O_2 for 4 hours at 70°C and counted by the addition of 10 ml of Triton/toluene scintillant (section 2.1.4).

2.4.2 Electrophoretic Analysis of Protein

2.4.2.1 Discontinuous SDS-polyacrylamide slab gel electrophoresis

Total, soluble and insoluble protein was monitored by SDS-polyacrylamide slab gel electrophoresis, using a modification of the method described by Laemmli (1970).

1) A discontinuous system was used with a stacking gel of 5% (w/v) acrylamide (0·8 mm x 25 mm x 145 mm) and a separating gel of 15% (w/v) acrylamide (0·8 mm x 110 mm x 145 mm). The following stock solutions were prepared and stored as indicated:

a) acrylamide stock solution — 30% (w/v) acrylamide, 0·2% (w/v) bis-acrylamide

filtered after preparation and stored in the dark at 4°C for up to 2 weeks.

b) stacking gel buffer (X 10) — 0·6 M Tris-HCl pH 6·8

stored at 4°C for up to 2 weeks

c) separating gel buffer (X 5) — 1·875 M Tris-HCl pH 8·8

stored at 4°C for up to 2 weeks

d) electrode buffer — 0·025 M Tris base, 0·192 M glycine

0•1% (w/v) SDS

freshly prepared

e) sample buffer — 60 mM Tris–HCl pH 6•8

2% (w/v) SDS

5% (w/v) 2ME

10% (w/v) sucrose

prepared at double strength, bromophenol blue added, and stored frozen at $-20^{o}C$.

The final composition of the gels was:

Stacking gel	Separating gel
5% (w/v) acrylamide	15% (w/v) acrylamide
0•033% (w/v) bis-acrylamide	0•1% (w/v) bis-acrylamide
0•06 M Tris–HCl pH 6•8	0•375 M Tris–HCl pH 8•8
0•1% (w/v) SDS	0•1% (w/v) SDS
0•1% (v/v) TEMED	0•5% (v/v) TEMED
0•03% (w/v) AMPS	0•145% (w/v) AMPS

The gel mixtures were de-gassed before addition of SDS, TEMED and AMPS. The separating gel was poured into prepared glass plates and overlaid with water to form a flat surface and allow anoxic polymerisation. The water overlay was removed after 1 hour, the gel surface rinsed with a small volume of unpolymerised stacking gel, the stacking gel poured and a slotted comb inserted. The comb was removed after 45 min and the sample wells rinsed twice with electrode buffer to remove any unpolymerised gel. The bottom spacer was removed from the plates, the plates inserted into the electrophoresis tank and the sample wells filled with electrode buffer. The upper and lower reservoirs of the tank were filled with electrode buffer.

2) The protein samples were heated to 70°C for 15 min and loaded into the wells. Electrophoresis was carried out at room temperature at a constant current of 15 mA per gel until the bromophenol blue reached the bottom of the gel.

3) The gel was fixed and stained in 0·1% (w/v) Coomassie brilliant blue R in 50% (v/v) methanol, 7% (v/v) acetic acid for 30 min, and destained in 3 changes of destain (25% (v/v) ethanol, 7% (v/v) acetic acid). The gel was dried down under vacuum onto a piece of Whatman 3MM chromatography paper. Standard molecular weight markers were used on each gel to determine the molecular weights of the polypeptides (Weber and Osborn, 1975; Kopperschläger et al., 1969).

bovine serum albumin	68,000
catalase (bovine liver)	60,000
aldolase	40,000
carbonic anhydrase	29,000
trypsin inhibitor (soybean)	21,000
myoglobin	17,200
lysozyme	14,300

2.4.2.2 IEF-SDS-polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis of protein was carried out as described by O'Farrell (1975) with several modifications. This method gives high resolution and sensitivity using separation by 2 independent parameters. Proteins were separated according to isoelectric point by isoelectric focusing in the first dimension and according to molecular weight by SDS-polyacrylamide gel elctrophoresis in the second dimension. The gel used for isoelectric focusing has large pores so as not to impede the

progress of the proteins through the pH gradient. Ampholines are electrophoresed through the gel prior to sample addition to establish the pH gradient. As protein moves down the tubes it encounters a higher and higher pH environment. As the protein traverses the pH gradient it reaches a pH corresponding to its isoelectric point. At this pH, net charge of protein is zero and it therefore stops moving in the electric field.

1st dimension-isoelectric focusing

1) The following solutions were used:

A. sample buffer	9•5 M urea
	2% (w/v) NP-40
	5% (w/v) 2ME
	2% (w/v) ampholines, pH 3•5-10
	0•01 M Na phosphate buffer, pH 6•7 (Documenta Geigy, p.281)
	1 mM EDTA, Na_2
D. IEF acrylamide stock	29•2% (w/v) acrylamide
	0•8% (w/v) bis-acrylamide
H. gel overlay	8 M urea
K. sample overlay	8 M urea
	1% (w/v) ampholines, pH 3•5-10
O. SDS buffer	10% (w/v) glycerol
	5% (w/v) 2ME
	2•3% (w/v) SDS
	0•0625 M Tris-HCl pH 6•8

Solutions A, H and K were stored frozen at -20°C and D and O were stored at 4°C for up to 2 weeks. Solution D was filtered and stored in the dark.

IEF gels were prepared in silanised glass tubes (2·5 mm x 130 mm). The bottom of the tubes were sealed with 2 layers of parafilm, and the gel mixture was prepared as follows:

urea	5·5 g
solution D	1·33 ml
water	2·0 ml
10% (w/v) NP-40	2·0 ml
ampholines, pH 3·5-10	0·5 ml
••••••••••degas twice••••••••••	
TEMED	7 µl
AMPS (10% (w/v))	10 µl

The gels were poured and overlaid with 20 µl of gel overlay H. Polymerisation was allowed to proceed for 2 hours, whereupon the gel overlay H was removed and replaced with 20 µl of sample buffer A for a further hour. 20 µl of fresh sample buffer A was overlaid, the gel tubes filled to the top with 0·2% (v/v) H_2SO_4 and the gels pre-run for 15 min at 200 V, 15 min at 300 V and 30 min at 400 V (constant voltage), using 0·2% (v/v) H_2SO_4 as anode buffer and 0·5% (v/v) ethanolamine as cathode buffer.

The overlay was removed, the samples loaded into the tubes with an overlay of 10 µl of sample overlay K, and the tubes filled with anode buffer. Fresh anode buffer was used for focusing and the gels were run at 200 V for 30 min, 300 V for 30 min and 400 V for 20 h at a constant temperature of 20°C. After focusing, the gels were removed from the tubes by gentle air pressure and equilibrated in 5 ml of SDS buffer O for 90 min to remove the ampholines and replace the urea with SDS. The gels were frozen at -80°C in aluminium troughs, and stored until required for up to 4 weeks. A blank gel

was included in each set, loaded with 20 µl of sample buffer A, which was run to determine the pH gradient. This gel was cut into 0·5 cm segments (without prior equilibration) and each segment was incubated in 2 ml of water for 4 hours. The pH of the solution was measured with an E.I.L. pH meter, Model 38A.

2) Protein samples were acetone precipitated from grinding buffer (section 2.3.5.2) and resuspended in sample buffer A. Routinely up to 200 µg of protein could be loaded in up to 50 µl of A.

2nd dimension-SDS-polyacrylamide slab gel electrophoresis

1) The following solutions were used:

L. separating gel buffer	1·5 M Tris-HCl pH 8·8
	0·4% (w/v) SDS
M. stacking gel buffer	0·5 M Tris-HCl pH 6·8
	0·4% (w/v) SDS
N. slab gel acrylamide stock	23·38% (w/v) acrylamide
	1·62% (w/v) bis-acrylamide
R. electrode buffer	0·025 M Tris base
	0·192 M glycine
	0·1% (w/v) SDS

Solutions L, M and N were stored at $4^{o}C$ for up to 2 weeks, solution N was filtered and kept in the dark, and solution R was freshly prepared.

Focused polypeptides were further separated according to their molecular weight by electrophoresis on a discontinuous gradient SDS slab gel. The apparatus used was made by the Botany Department workshop; the upper reservoir contacted the top of the gel directly (thus eliminating the need for a wick), and the glass plates were

modified (O'Farrell, 1975) to contain a notch. The separating gel (0•8 mm x 110 mm x 145 mm) was prepared as a 10•5% to 15% exponential gradient using the following acrylamide mixtures:

Component	weak acrylamide	strong acrylamide
separating gel buffer L	3•75 ml	1•5 ml
acrylamide stock N	5•25 ml	3•0 ml
water	6•00 ml	-
75% (w/v) glycerol	-	1•5 ml
10% (w/v) AMPS	21•75 µl	8•7 µl
TEMED	7•5 µl	3•0 µl

The gel was overlaid with water and allowed to polymerise for 1 hour. The water was removed and the gel surface washed overnight with a few ml of 4X diluted buffer L. Buffer L was removed and the gel surface washed with a small volume of unpolymerised stacking gel. The stacking gel (0•8 mm x 25 mm x 145 mm) was poured, a flat surface made with a perspex spacer and the gel was allowed to polymerise for 45 min:

stacking gel buffer M	2•5 ml
acrylamide stock N	1•5 ml
water	6•0 ml
10% (w/v) AMPS	30 µl
TEMED	10 µl

•••••••sufficient for 2 slab gels•••••••

Agarose was dissolved in SDS buffer O (1% w/v) by heating to 90^{o}C and subsequent cooling to 50^{o}C. The agarose solution was poured onto the top of the slab after any unpolymerised gel had been removed

from the top of the stacking gel. Excess liquid was removed from the thawed IEF gel, and the IEF gel rolled into position into the agarose. Electrophoresis was carried out at 10 mA per gel until the bromophenol blue (added to the upper reservoir) reached the separating gel. The current was then increased to 15 mA per gel until the tracker dye reached the bottom of the gel.

2) After separation in the 2nd dimension, the gels were fixed and stained for 30 min and destained for 3 hours (section 2.4.2.1.3). The gels were dried down under vacuum onto 3MM chromatography paper.

2.4.3 Fractionation of Nucleic Acids

Phenol-detergent extracted nucleic acid preparations were fractionated on 2·4% cylindrical polyacrylamide gels by the method of Loening (1967) as modified by Leaver (1973).

acrylamide stock solution
15% (w/v) acrylamide
0·75% (w/v) bis-acrylamide
filtered and stored in the dark at $4^{o}C$.

electrophoresis buffer (5X)
43·5 g Tris base
46·8 g $NaH_2PO_4 \cdot 2H_2O$
3·7 g EDTA.$Na_2 \cdot 2H_2O$
diluted 5 fold and made 0·05% (w/v) with respect to SDS for use as electrode buffer

gel mixture
5 ml acrylamide stock solution
6·25 ml electrophoresis buffer (5X)
19·7 ml water
250 µl 10% (w/v) AMPS
25 µl TEMED

Gels (8 cm x 0·7 cm) were overlaid with water, allowed to polymerise for 30 min, and kept overnight at 4°C. The gels were pre-run for 30 min at 50 V per tank of 8 gels at 4°C, to remove the catalysts and allow SDS into the gel. Samples of up to 25 µg of nucleic acid were loaded onto the gels in electrode buffer made 5% (w/v) with respect to sucrose. Electrophoresis lasted for 3·5 hours at 4°C.

Gels were removed after electrophoresis and either soaked for 1 hour in water to remove any UV absorbing background material, prior to scanning the gels at 265 nm using a Joyce Loebel UV scanner; or stained in 1µg. ml^{-1} ethidium bromide for 1 hour and photographed using UV optics.

2.5 METHODS (LABELLING)

2.5.1 Quantitative Estimation of In Vivo DNA and RNA Synthesis

DNA and RNA were assayed by a modification of the method of Guinn (1966), and the RNA and DNA content and specific activity estimated after alkaline and acid hydrolysis (Schmidt and Thannhauser, 1945).

2.5.1.1 Extraction

Fronds were incubated in either ^{3}H thymidine or ^{3}H uridine (5 µCi. ml^{-1}) in H/2 for 3 hours, washed with water, and homogenised in 5 ml of 76% (v/v) ethanol, 2% (w/v) NaCl. The resultant homogenate was sampled for isotope uptake, and centrifuged at 10,000 g_{av} for 15 min. The pellet was resuspended in ethanol-NaCl and incubated at 70°C for 10 min. The pellet was re-extracted in this way until a colourless supernatant was achieved and then resuspended in 5 ml of 10% (w/v) NaCl and incubated for 1 hour at

100°C. The supernatant was retained and the pellet re-extracted in 10% (w/v) NaCl. The supernatants were pooled and the nucleic acids precipitated by the addition of 2 ml of 20% (w/v) TCA and left on ice for 1 hour. The precipitate was harvested at 25,000 g_{av} for 10 min and washed with 5 ml of 0·2 M PCA.

2.5.1.2 Hydrolysis of RNA and DNA

While acid hydrolysis was all that was necessary to solubilise both RNA and DNA for content measurement, differential hydrolysis was necessary so that the radioactivity associated with both fractions could be calculated. This provided a check on any non-specific labelling that might have occurred.

The precipitate was dissolved in 1 ml of 0·1 M NaOH at 30°C overnight to solubilise RNA; DNA was solubilised by the addition of 1 ml of 1 M PCA and incubation at 70°C for 30 min. A standard solution of calf thymus DNA (100 $\mu g.\ ml^{-1}$) was simultaneously hydrolysed in 0·5 M PCA since acid hydrolysis of DNA is time dependent. Samples were cooled in iced water and centrifuged at 1,000 g_{av} for 10 min. Aliquots of the resultant supernatant were assayed for nucleic acid content by the determination of the OD_{260} (assuming that an OD of 1 corresponds to a nucleic acid concentration of 40 $\mu g.\ ml^{-1}$). Radioactivity incorporated into either DNA or RNA was determined by counting samples of the hydrosylates in Triton/ toluene scintillant. DNA content was measured by the diphenylamine assay (Burton, 1956).

2.5.1.3 Diphenylamine assay

Reagent: 1·5 g of diphenylamine was dissolved in 100 ml of glacial acetic acid and 1·5 ml of concentrated sulphuric acid. 0·5 ml of

aqueous acetaldehyde (16 mg. ml^{-1}) was added immediately before use.

1 ml samples containing between 1 and 50 µg of hydrolysed DNA were added to 2 ml of reagent, mixed and incubated for 18 hours at 25°C. The OD was read at 600 nm, and the DNA content calculated by comparison with the standard curve. RNA content was calculated by subtracting the DNA content from the total nucleic acid content.

2.5.2 Quantitative Estimation of In Vivo Protein Synthesis

Fronds were incubated with ^{35}S methionine (2 µCi. ml^{-1}) for 3 hours. The tissue was washed and homogenised in 2 ml of 0·1 M NaOH to ensure complete homogenisation. Duplicate 10 µl samples of the homogenate were counted in Triton/toluene scintillant to calculate the amount of radioactivity absorbed into the tissue. The homogenate protein was precipitated with an equal volume of 10% (w/v) TCA and left on ice overnight. The precipitate was recovered by centrifugation at 10,000 g_{av} for 15 min, washed twice with 5% (w/v) TCA and once in hot methanol. The pellet was resuspended in 0·1 M NaOH and incubated at 37°C overnight. Aliquots of the hydrosylate were taken for protein estimation (section 2.3.5.3) and for counting in Triton/toluene scintillant to calculate the radioactivity incorporated into protein. Protein synthesis was estimated as cpm incorporated. µg $protein^{-1}$. 10^6 cpm $absorbed^{-1}$.

2.5.3 Qualitative In Vivo Labelling of Protein

Qualitative developmental changes in protein synthesis were studied by *in vivo* pulse labelling of the tissue with ^{35}S methionine. Although this method was designed primarily to study changes in the electrophoretic pattern of the polypeptides, quantitative estimates

of soluble and insoluble protein synthesis could be calculated.

1) Fronds were incubated with ^{35}S methionine (section 2.5.2), but the fronds were homogenised in grinding buffer (section 2.3.1). The homogenate was centrifuged at 10,000 g_{av} for 10 min and samples of the supernatant taken for protein assay (sections 2.3.5.3 & 2.3.5.4). The remaining supernatant was stored at -40°C until required for electrophoretic fractionation (section 2.4.2). Where insoluble proteins were to be studied, the pellet proteins were also stored frozen after washing and solubilisation (section 2.3.5.4).

2) Radioactive polypeptides were detected on gels by autoradiography or fluorography. Dried down gels were autoradiographed for up to 2 weeks (depending on the amount of radioactivity incorporated into protein) by exposing to Blue Brand X-ray film at room temperature.

For fluorography, gels were impregnated with Enhance and washed in water to precipitate the scintillant and rehydrate the gel. Gels were dried down as above. Sensitivity of the fluorographic technique was improved by flashing X-omatic H film from a height of 25 cm with a Sun-Pak GT 32 flash gun for 1/1,000 s, through an Ilford S902 filter covered with a sheet of Whatman no. 1 paper. The sensitised side of the film was placed against the gel, and the gel exposed for up to 2 weeks at -80°C. This procedure (Bonner and Laskey, 1974) sensitises the film by the production of a silver atom from a silver ion; low temperature prevents the thermal reversion to silver ion. A grain of silver is formed only when a second silver atom is produced by the emission of a β-particle from the radioactive source.

X-ray film was developed for 4 min in Phen-X developer, stopped

in 2% (v/v) acetic acid and fixed in IF 23, before washing and drying. Photographs were taken of the autoradiographs and fluorographs using a light box and a Cosina CSM camera, loaded with Pan F film rated at 100 ASA. Film was developed in Microphen developer for 7 min.

2.5.4 In Vitro Protein Synthesis

2.5.4.1 Preparation of wheat-germ S30

A wheat-germ S30 cell free protein synthesising system was prepared by the method of Roberts and Paterson (1973) as modified by Weir et al. (1980). All steps of the procedure were carried out at $4^{o}C$.

5 g of wheat-germ was ground to a fine powder with an equal volume of powdered glass using a mortar and pestle. 13 ml of buffer (20 mM Tris-Ac pH 7·6; 120 mM KAc; 5 mM $MgAc_2$; 1 mM DTT) was added and homogenisation continued until a smooth paste was obtained. The homogenate was centrifuged at 30,000 g_{av} for 15 min, and the resultant supernatant loaded onto a Sephadex G25 column which had been equilibrated with buffer. 1 ml fractions of the eluate were collected, and those from the first peak, having an OD_{260} of $\geq$100 units. ml^{-1} were combined. This fraction was centrifuged at 30,000 g_{av} for 15 min and the supernatant dialysed overnight (Zagórski, 1978) with 20 mM HEPES-KOH pH 7·6, 120 mM KAc, 5 mM $MgAc_2$, 6 mM 2ME. After dialysis, the preparation was centrifuged as before and the final supernatant stored frozen in 250 µl aliquots at $-80^{o}C$.

2.5.4.2 Characterisation of the wheat-germ system

The wheat-germ system was used as an assay for 'translatable'

mRNAs in different nucleic acid preparations. Incubations were carried out in a total volume of 50 µl containing the components listed below:

a) wheat-germ S30	1 OD_{260} unit
b) HEPES-KOH	25 mM
potassium acetate	94 mM
magnesium acetate	2 mM
spermidine	0·25 mM
ATP	1 mM
GTP	50 µM
creatine phosphate	8 mM
creatine kinase	5 µg
DTT	2 mM
amino acids (excluding methionine)	25 µM each
^{35}S methionine	5 µCi
c) nucleic acid	6 µg

Components a and b were contained in 20 µl of the incubate while the remaining 30 µl contained the nucleic acid and any sterile water required to bring the volume of the incubate up to 50 µl. Incubations were carried out at 25°C for 90 min and a time course of incorporation of ^{35}S methionine into protein was monitored by taking 5 µl samples at 10, 30, 60 and 90 min. The samples were spotted on 3MM filter paper discs and processed by the method of Mans and Novelli (1961) which determines the radioactivity precipitable in hot TCA. The amount of radioactivity was determined by counting each disc in 5 ml of Butyl-PBD scintillant (section 2.1.4). Duplicate samples of the original incubation mixture were taken prior

to incubation, and either treated by the Mans and Novelli procedure, or counted directly. These showed respectively, the amount of radioactivity not due to labelled protein and not washed off the filter, and the amount of radioactivity supplied to each incubation. A control incubation to which no exogenous nucleic acid had been added was included, so that the level of incorporation due solely to endogenous mRNA could be determined.

2.5.4.3 Fractionation of in vitro translation products

The products synthesised in the wheat-germ system were precipitated with 4 volumes of acetone and kept at $0^{o}C$ for at least 30 min. The precipitated protein was pelleted by centrifugation at 12,000 g_{max} for 1 min, then resuspended in gel sample buffer for SDS PAGE (section 2.4.2.1) or sample buffer A for 2-dimensional analysis (section 2.4.2.2). The gels were stained, destained, and fluorographed (section 2.5.3.2).

2.5.4.4 Optimisation of the wheat-germ system

The wheat-germ system used to compare the translatable mRNA content during turion formation was initially optimised for S. polyrrhiza RNA, so that high levels of incorporation and stimulation above the endogenous background, and high molecular weight products could be obtained.

Variations in the concentrations of wheat-germ S30, potassium, magnesium and exogenous nucleic acid were studied. The following concentration ranges were studied:

a) wheat-germ S30	0·5 - 2·0 OD_{260} units
b) potassium	84 - 124 mM
c) magnesium	1·75 - 2·75 mM

d) nucleic acid 2·5 - 50 µg

2.5.5 Compartmental Analysis of Ion Flux and Content in Vegetative Fronds and Turions of Spirodela polyrrhiza

Compartmental analysis of radioisotope elution measurements has become a well established method of estimating unidirectional fluxes of ions across cell membranes in higher plants (Walker and Pitman, 1976). Together with transmembrane electrical potential values, these measurements can yield information about ion absorption at the cellular level which is not possible to obtain directly, mainly due to the minute volumes of the individual compartments involved. Difficulties arise from differentiation in higher plant tissue and results obtained from uniform populations of cells (e.g. oat coleoptiles; Pierce and Higinbotham, 1970) inspire the most confidence. However, in this preliminary study of ion fluxes in S. polyrrhiza fronds and turions, fraught as it is with both practical and theoretical problems, cell differentiation is a problem that cannot readily be side-stepped.

The procedure for calculating ion flux is based on the determination of rate constants for exchange of tracer between tissue and solution, and on the use of these rate constants to find values for efflux, influx, and content for the particular ion in question. The theory of tracer measurement on compartmental systems is covered in Sheppard's book (1962), the mathematical techniques appropriate to the extraction of values from the data are introduced by Magar (1972), and the plant literature is reviewed by MacRobbie (1977).

Fronds and turions of Spirodela polyrrhiza (N) were rinsed briefly in 1 mM KCl, 1 mM $Ca(NO_3)_2$, 0·25 mM $MgSO_4$, 0·904 mM NaH_2PO_4, 0·048 mM Na_2HPO_4 (Macklon, 1975a), and then incubated in this medium (1X) in petri-dishes for 1 hour at 25°C. This was replaced by 5 ml of fresh medium (experimental solution). Some experiments were performed using H/2 throughout. All steps up to elution were performed using aseptic techniques, and all solutions (including elution) were autoclaved.

2.5.5.1 Flux experiments

For each ion studied isotope was incorporated into the experimental solution as shown below:

a) $H^{36}Cl$ (89·9 μCi. ml^{-1}; 7 mg Cl. ml^{-1}) was added at 0·456 μCi. ml^{-1} (i.e. 1 mM HCl). The medium was adjusted prior to isotope addition, so that all the Cl was provided by the isotope, and then was neutralised with 1 mM KOH to return the pH to 5·6.

b) $^{22}NaCl$ (200 μCi. ml^{-1}; 1·9 μg Na. ml^{-1}) was added at 1 μCi. ml^{-1} which had a negligible effect on the total Na concentration.

c) ^{42}KCl (100 μCi. ml^{-1}; 11·5 mg KCl. ml^{-1}) was added at 0·64 μCi. ml^{-1} (i.e. 1 mM KCl). The medium was adjusted so that all the KCl was provided by the isotope.

d) $^{45}CaCl_2$ (1·46 mCi. ml^{-1}; 95 μg Ca. ml^{-1}) was added at 1 μCi. ml^{-1} which had a negligible effect on the total Ca concentration.

When the effect of short and long exposures to ABA was studied, the medium was made 10^{-7} M with respect to ABA either at the time of isotope addition (18 hours ABA), or at 1 hour before isotope elution (1 hour ABA). When ABA was added to vegetative fronds, it was also included in the elution medium (and indeed remained with the turions

at all times to prevent germination).

After 18 hours of isotope uptake, the uptake solution was removed with a syringe (with a sawn-off needle to recover every drop), and fresh medium added to the petri-dish. This medium was withdrawn 30 seconds later and transferred to a scintillation vial. The tissue was eluted in this way as follows:

every	0•5 min	up to	1•5 min
"	1•0 "	"	2•5 "
"	1•5 "	"	4•0 "
"	2•5 "	"	6•5 "
"	3•5 "	"	10 "
"	10 "	"	40 "
"	20 "	"	60 "
"	30 "	"	120 "
"	60 "	"	360 "
"	90 "	"	540 "

The tissue was transferred to a Whatman no. 541 filter paper after the elution and weighed. Hot water (analar) extracts were made of the labelled tissue for radioactive and chemical assay in the case of ^{22}Na, ^{36}Cl and ^{42}K. Each sample was extracted with 3 changes of boiling water (5 ml, 2•5 ml and 2•5 ml) for a total of 20 min. For ^{45}Ca the tissue was dried, ashed, taken up in 6M HCl and finally made up to volume with 0•1 M HCl for counting or chemical analysis. Chloride was estimated using the direct potentiometric method of Shone (1968), sodium and potassium by flame emission spectrophotometry and calcium by atomic absorption spectrophotometry. Radioactivity in uptake solutions, hot water

extracts, 0•1 M HCl extracts and in efflux washing solutions, was determined by mixing each 5 ml sample with an equal volume of Instagel scintillant. The samples of ^{42}K were counted in glass vials without scintillant, employing the Čerenkov principle.

2.5.5.2 Uptake experiments

Uptake was monitored in essentially the same manner as for flux experiments. Samples of tissue were loaded with isotope as follows:

a) 1 $\mu Ci.\ ml^{-1}$ of $^{45}CaCl_2$

b) 0•0456 $\mu Ci.\ ml^{-1}$ of $H^{36}Cl$

c) 0•1 $\mu Ci.\ ml^{-1}$ of $^{22}NaCl$

d) 0•064 $\mu Ci.\ ml^{-1}$ of ^{42}KCl

The time course of uptake was monitored by measuring the radioactivity (apparent uptake) and the chemical content (net uptake) for each sample of tissue at time intervals up to 30 hours after loading.

CHAPTER 3

RESULTS AND DISCUSSION

GROWTH AND ORGANISATION OF SPIRODELA POLYRRHIZA

3.1 GROWTH OF THE VEGETATIVE FROND OF SPIRODELA POLYRRHIZA

The growth of Spirodela polyrrhiza was investigated by measuring increase in frond number and by taking fresh and dry weights, since all three methods have been widely used in the literature to describe Lemnacean growth.

3.1.1 Growth Measurement by Frond Number

Initially a mother frond produces 2 daughter fronds, which in turn also produce 2 daughters each. The mother frond continues to produce daughters (Fig. 1). If S. polyrrhiza reproduced in this way continually, then growth could not be described as exponential in the way that bacterial growth is, since one would expect more than 8 fronds by the 3rd generation. A bacterium will continue to divide and produce essentially equivalent daughter cells with at least potentially unlimited capacities to divide. A bacterial cell divides into 2 cells which in turn divide to become 4 cells; after 3 generations all eight cells are of the same age, and none of the original cells is present. In a Spirodela culture however, each frond can only produce a certain number of daughters before it dies, and the daughter fronds produced by a young frond are not equivalent to those produced later by the same frond (Claus, 1972). The growth of the culture is essentially exponential under non-limiting conditions because there is a cycle of senescence as well as one of rejuvenation in each culture (Ashby and Wangermann, 1949).

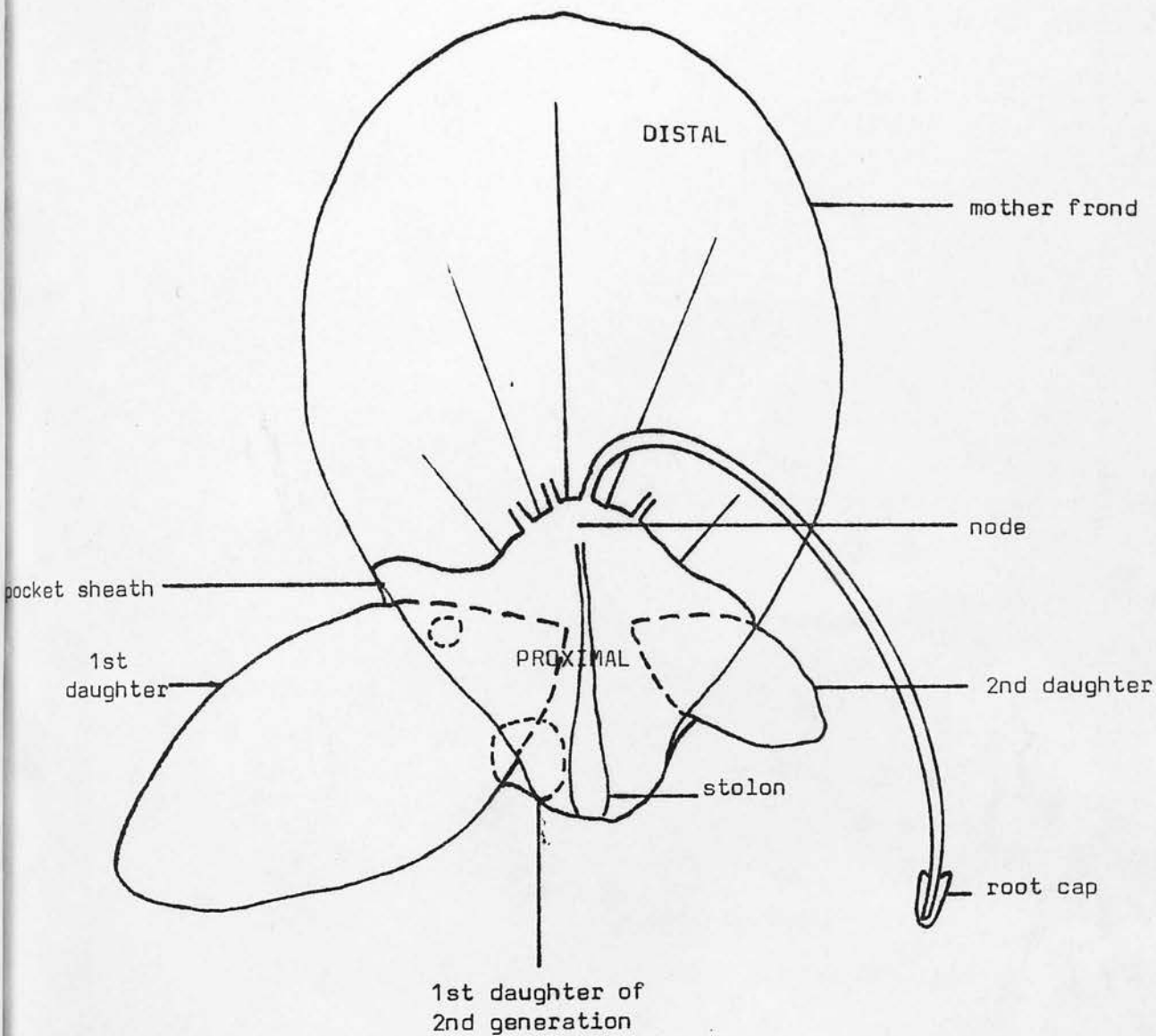
The growth of a culture is therefore an exponential function represented by the equation:

$$F_t = F_o\, e^{kt} \qquad \ldots\ldots\ldots\

Figure 1

Diagrammatic representation of the ventral view of a frond of Spirodela polyrrhiza, showing the mode of production of daughter fronds. Dotted lines show sub-surface structures. Only 1 root is shown for convenience. X 15 approximately.

FIGURE 1


DISTAL
mother frond
node
pocket sheath
1st
daughter
PROXIMAL
2nd daughter
stolon
root cap
1st daughter of
2nd generation

where, F_t = frond number at time t

F_o = original frond number

k = growth constant

and t = time in days.

Typical growth curves for both clones of S. polyrrhiza are shown in figure 2. Taking logarithms to the base 10:

$$\log F_t = \log F_o + kt \quad \ldots\ldots\ldots\

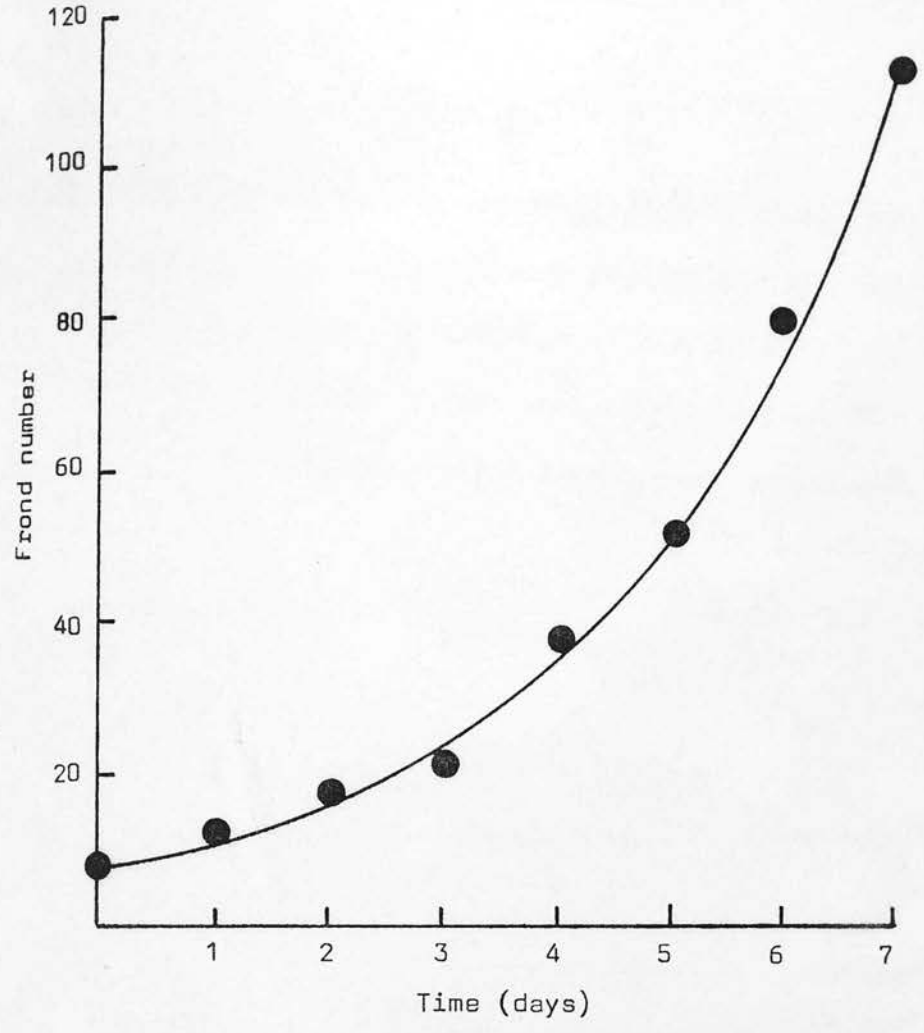
Figure 2

Daily growth of fronds of S. polyrrhiza growing in H/2 medium at 25°C. Growth was determined by counting all fronds visibly protruding from the mother pockets each day after innoculation of a plantlet onto fresh medium. Each value is the mean of 4 replicates.

a) S. polyrrhiza (UC)

b) S. polyrrhiza (N)

FIGURE 2


(A)
120
100
80
60
40
20
Frond number
1
2
3
4
5
6
7
Time (days)

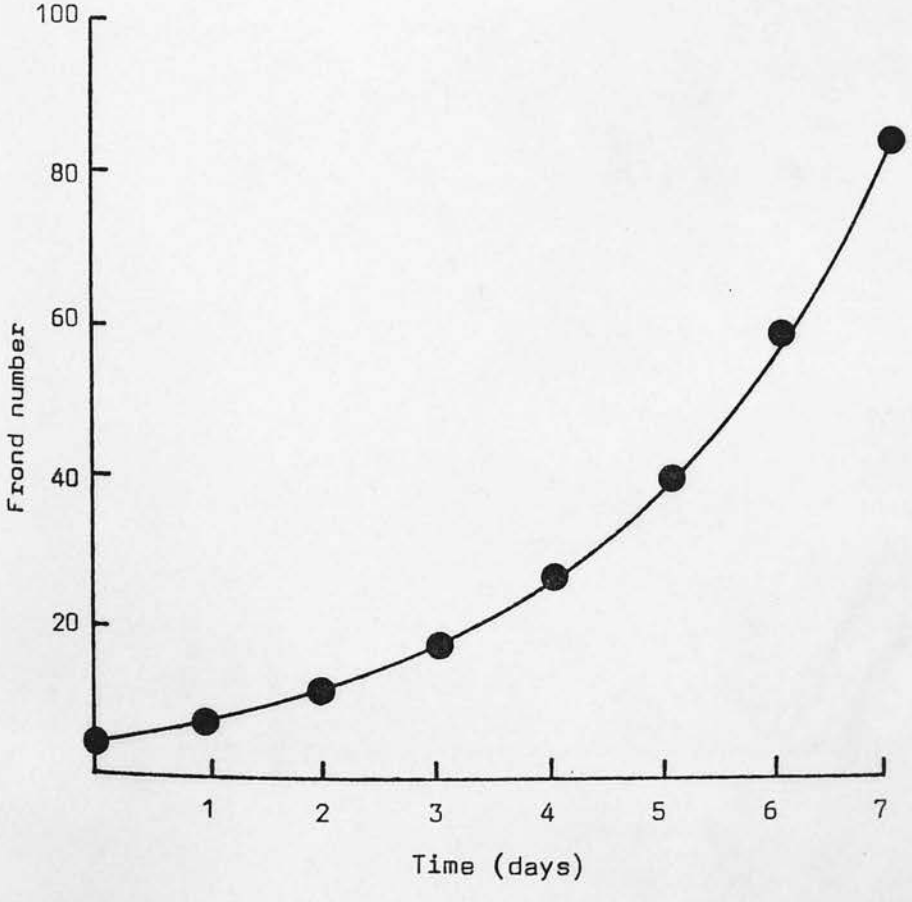

(B)
100
80
60
40
20
Frond number
1
2
3
4
5
6
7
Time (days)

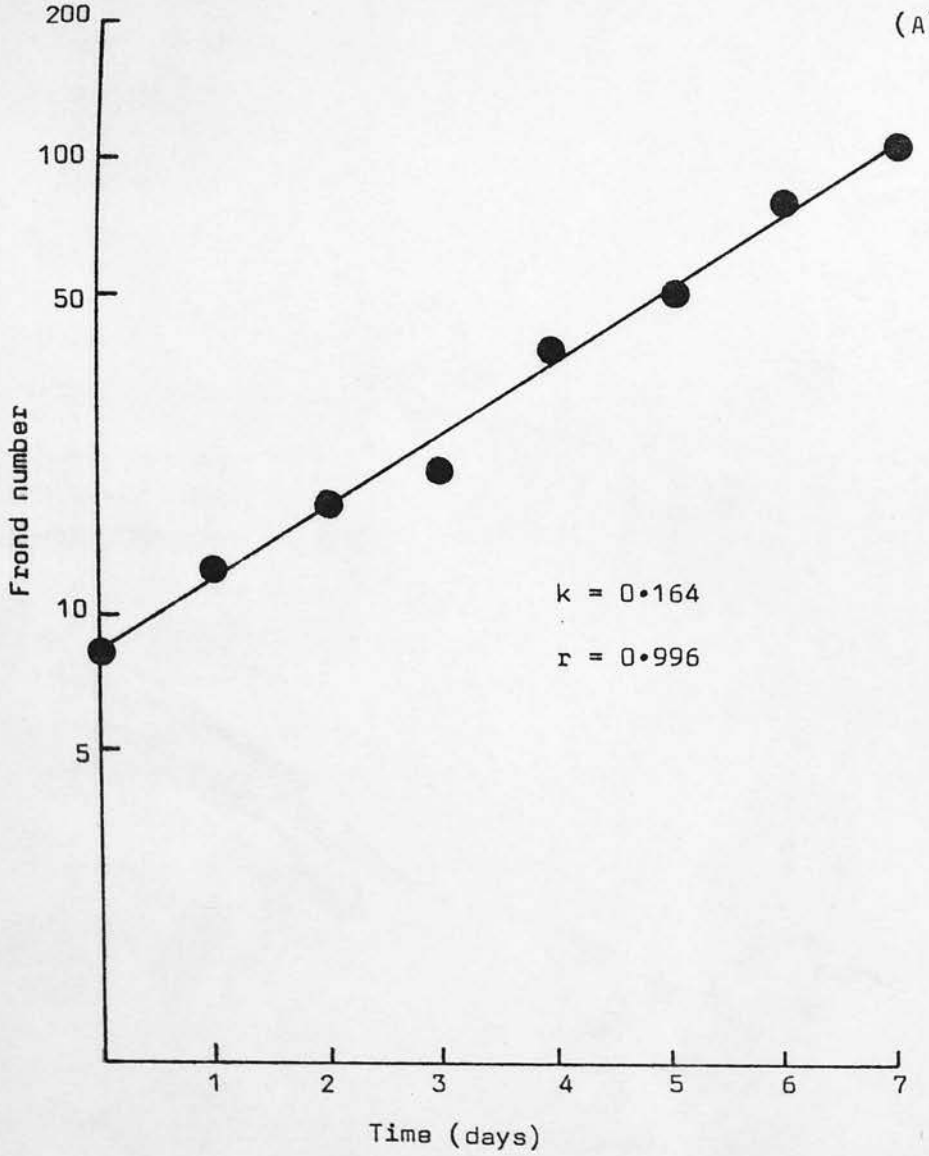
Figure 3

The exponential nature of growth of *S. polyrrhiza*. A plot of frond number (log) against time yields a staight line with a slope of k and an intercept of log F_o (the initial frond number). The lines were fitted by linear regression. Correlation coefficients (r) are shown on the figure. The higher the value of k (the growth constant) the faster the rate of growth.

a) *S. polyrrhiza* (UC)

b) *S. polyrrhiza* (N)

FIGURE 3


(A)
200
100
50
10
5
Frond number
k = 0•164
r = 0•996
1
2
3
4
5
6
7
Time (days)

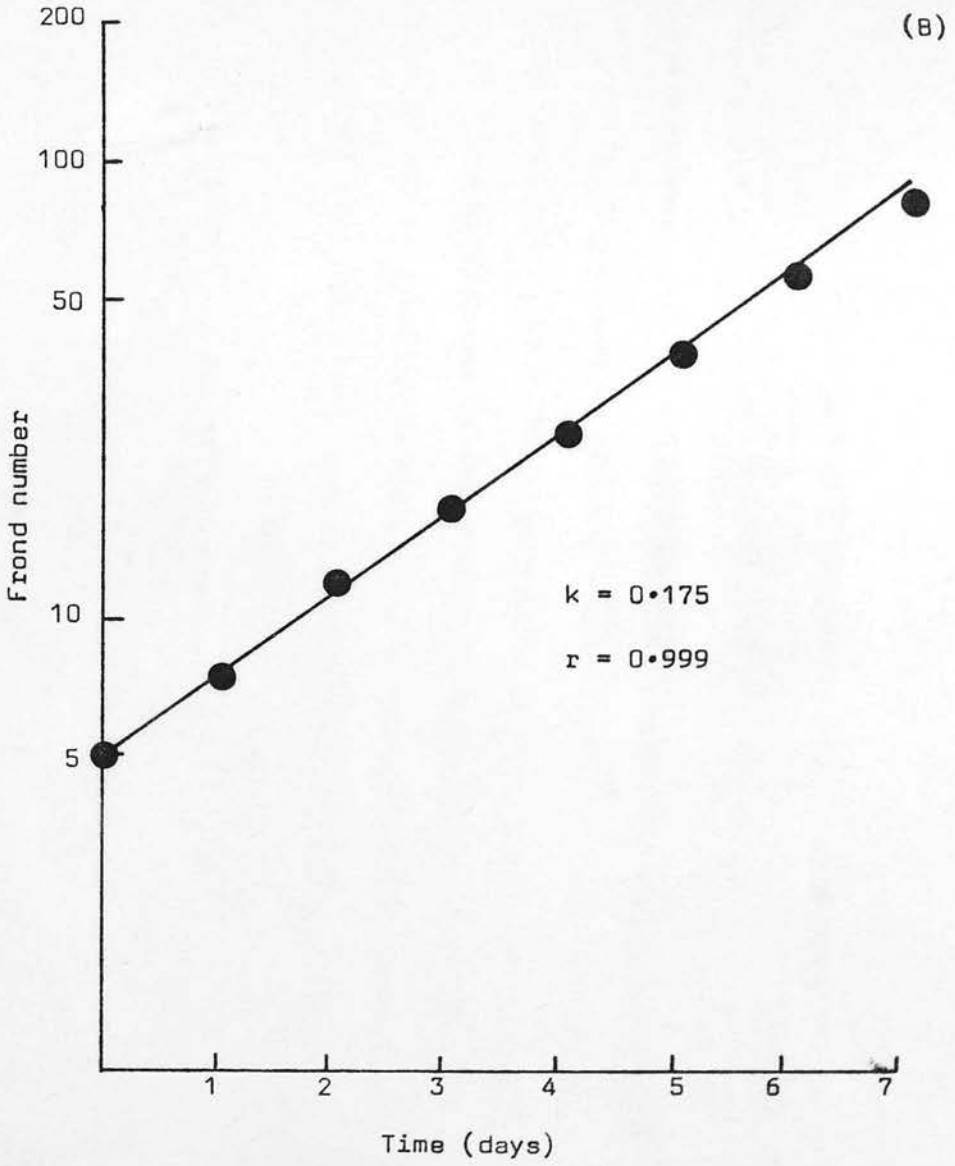

(B)
200
100
50
10
5
Frond number
k = 0•175
r = 0•999
1
2
3
4
5
6
7
Time (days)

Figure 4

The effect of ABA on the growth of S. polyrrhiza (UC). 8 fronds were innoculated onto H/2 medium supplemented with varying concentrations of ABA, and their daily growth was measured by counting frond number. The growth of each culture was plotted as in figures 2 and 3 and the linear regression of each treatment was calculated. All treatments resulted in linear regressions significant at the 1% level; correlation coefficients are shown below. The shaded area indicates the formation of turions.

Concentration of ABA	correlation coefficient r	Doubling time (days)
1×10^{-9}M	0.996	1•84
5×10^{-9}M	0•996	1•91
1×10^{-8}M	0•994	1•84
5×10^{-8}M	0•993	2•06
$7•5 \times 10^{-8}$M	0•999	3•34
1×10^{-7}M	0•988	4•33
$2•5 \times 10^{-7}$M	0•998	5•06
5×10^{-7}M	0•990	5•50
$7•5 \times 10^{-7}$M	0•982	9•26
1×10^{-6}M	0•995	8•14
$2•5 \times 10^{-6}$M	0•962	16•01
1×10^{-5}M	0•846	45•60

FIGURE 4

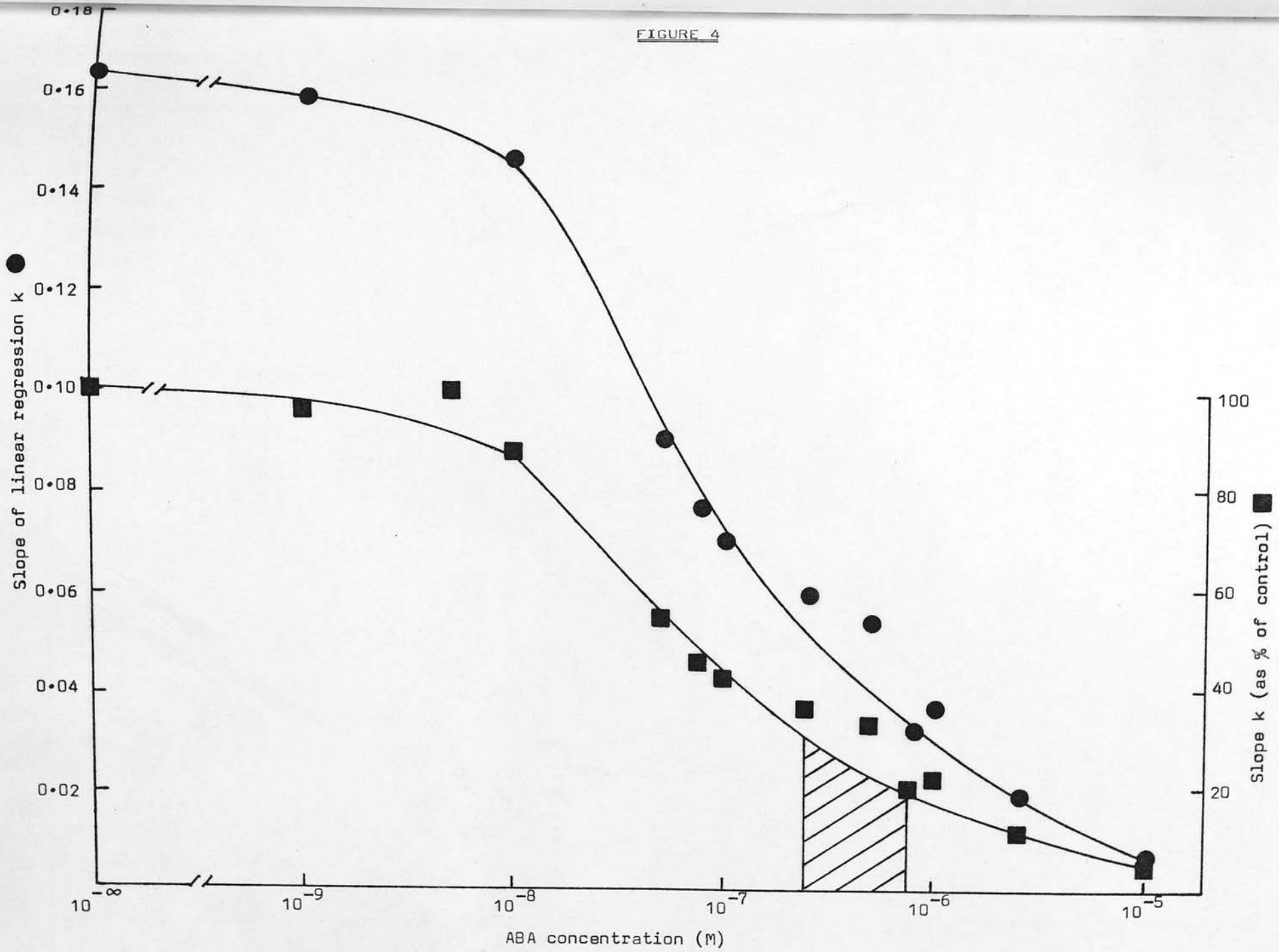
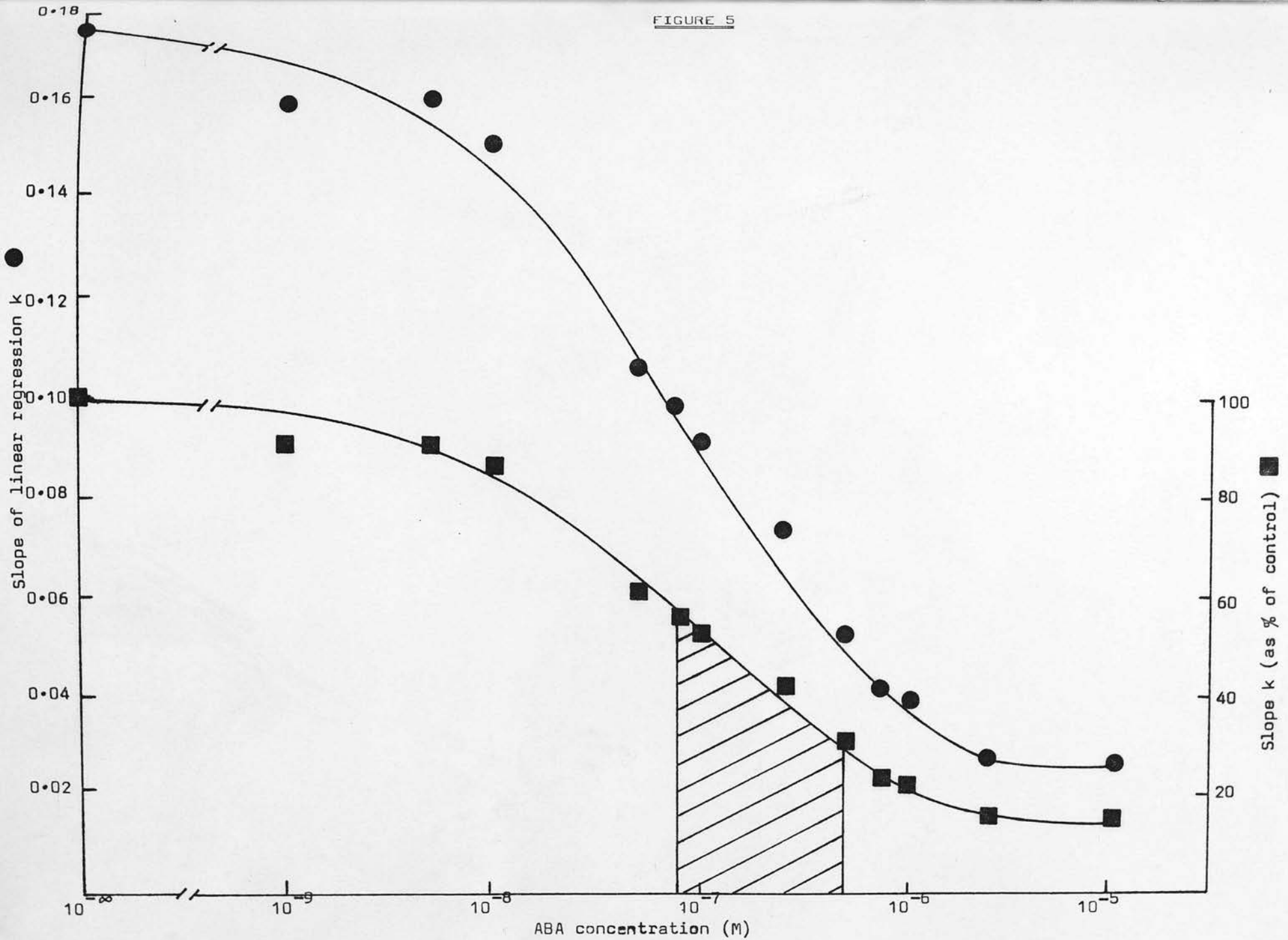

0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
Slope of linear regression k ●
100
80
60
40
20
Slope k (as % of control) ■
10^-∞
10^-9
10^-8
10^-7
10^-6
10^-5
ABA concentration (M)

Figure 5

The effect of ABA on the growth of S. polyrrhiza (N). 5 fronds were innoculated onto H/2 medium supplemented with varying concentrations of ABA, and their daily growth was measured by counting frond number. The growth of each culture was plotted as in figures 2 and 3 and the linear regression of each treatment was calculated. All treatments resulted in linear regressions significant at the 1% level; correlation coefficients are shown below. The shaded area indicates the formation of turions.

Concentration of ABA	correlation coefficient r	Doubling time (days)
1×10^{-9}M	0·999	1·90
5×10^{-9}M	0·998	1·89
1×10^{-8}M	0·999	2.01
5×10^{-8}M	0·981	2·84
$7{\cdot}5 \times 10^{-8}$M	0·993	3·07
1×10^{-7}M	0·991	3·29
$2{\cdot}5 \times 10^{-7}$M	0·977	4·09
5×10^{-7}M	0·971	5·73
$7{\cdot}5 \times 10^{-7}$M	0·955	7·25
1×10^{-6}M	0·930	7·64
$2{\cdot}5 \times 10^{-6}$M	0·895	11·07
1×10^{-5}M	0·899	11·07

FIGURE 5


0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
Slope of linear regression k
100
80
60
40
20
Slope k (as % of control)
10⁻∞
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
ABA concentration (M)

almost completely inhibited at 10^{-5} M. S. polyrrhiza (UC) was slightly more sensitive to ABA at the higher concentrations, growth being inhibited by 96% at 10^{-5} M in this clone compared with 85% inhibition in S. polyrrhiza (N) at the same concentration of ABA.

Stewart (1969) reported growth inhibition of S. polyrrhiza at concentrations of ABA as low as $5{\cdot}68 \times 10^{-10}$ M, although growth inhibition is not usually encountered in other strains and species at this concentration. Many authors have found that low concentrations of ABA do in fact stimulate growth. Stimulation of the growth of Lemna gibba was achieved with 10^{-8} M ABA as measured by frond number (Chen and Park, 1976) and between 10^{-11} and 10^{-8} M ABA when measured as fresh weight increase (Al-Shalan and Kandeler, 1978). ABA promoted an increase in frond number and fresh weight in S. polyrrhiza when applied at 10^{-10} to 10^{-8} M with maximum stimulation at 10^{-9} M (McWha and Jackson, 1976). No such stimulation of growth was found in either clone of S. polyrrhiza in this study, although concentrations of ABA $< 10^{-9}$ M were not investigated.

The growth inhibition found in this work (with 1×10^{-7} M to 5×10^{-7} M ABA producing a 50% response) is of the same order of magnitude as shown by most other Lemnaceae (Eliasson and Tillberg, 1976; van Staden and Bornman, 1969, 1970a; van Overbeek et al., 1967; McLaren and Smith, 1976; Newton, 1977).

3.1.3 Growth Measurement by Fresh and Dry Weight

The percentage growth inhibition was also estimated by measuring the increase in the fresh and dry weights of cultures

exposed to different ABA concentrations after 8 days growth. In S. polyrrhiza (UC) (fig. 6a) it is apparent that the % inhibition obtained with 10^{-5} M ABA as measured by fresh weight is identical to that obtained by frond number measurement (fig. 4). However, fresh weight determination was more sensitive in detecting growth inhibition at low concentrations of ABA, since fronds formed in the presence of ABA were often smaller than untreated fronds. Far less % growth inhibition was detected by dry weight measurement (76% at 10^{-5} M), probably due to ABA induced starch accumulation (McLaren, 1976). The effect of ABA on the fresh and dry weights of cultures of S. polyrrhiza (N) are shown in figure 6b.

3.2 GROWTH OF THE TURION OF SPIRODELA POLYRRHIZA

3.2.1 Abscisic Acid and Turion Production

Over a narrow concentration range, ABA not only inhibited the overall rate of growth of S. polyrrhiza, but induced the formation of turions (Plate 2). In S. polyrrhiza (UC) turions were produced only between $2{\cdot}5 \times 10^{-7}$ and $7{\cdot}5 \times 10^{-7}$ M ABA, and were recognisable as turions 5 days after ABA addition, although they were not fully mature (as judged by time of abscission from the mother frond) until 10 days. Mature turions were produced by S. polyrrhiza (N) within 7 days at concentrations of ABA between $7{\cdot}5 \times 10^{-8}$ and 5×10^{-7} M i.e. the turions of S. polyrrhiza (N) were formed more quickly and at lower ABA concentrations than those of S. polyrrhiza (UC).

Indirect evidence of the production of turions at these ABA concentrations can be seen in figures 4 and 5, where there is a slight resurgence of the growth rate at the concentrations of ABA where turions are produced. Turions were never produced without the

Figure 6

The effect of ABA on the growth of S. polyrrhiza as measured by fresh and dry weight. Plantlets were innoculated onto H/2 medium supplemented with various concentrations of ABA and the fresh and dry weights of the cultures were measured after 8 days incubation at 25°C. Values are the means of 4 replicates, and are expressed as a % of the control value (i.e. no ABA).

● fresh weight of culture

○ dry weight of culture

a) S. polyrrhiza (UC)

b) S. polyrrhiza (N)

FIGURE 6a

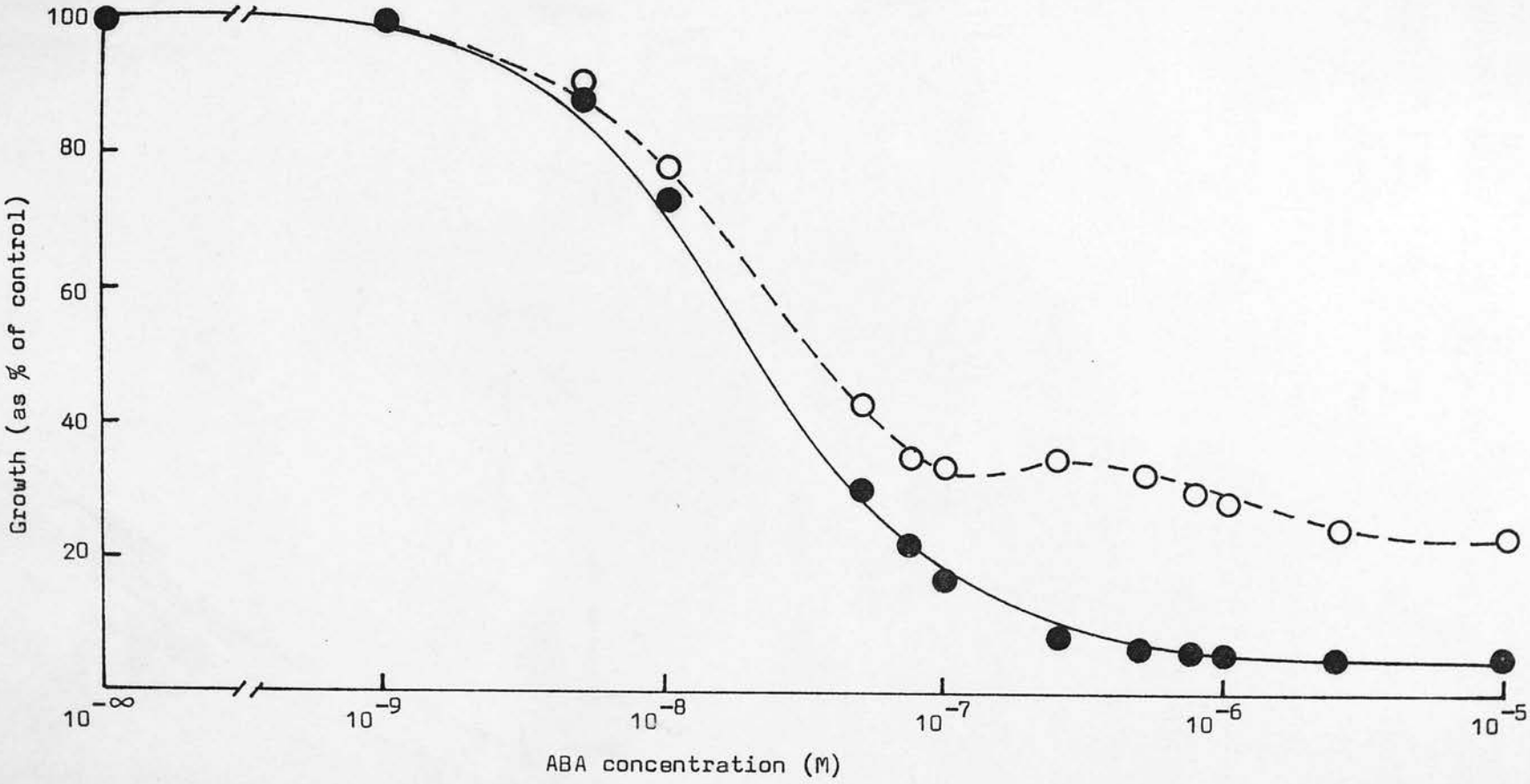
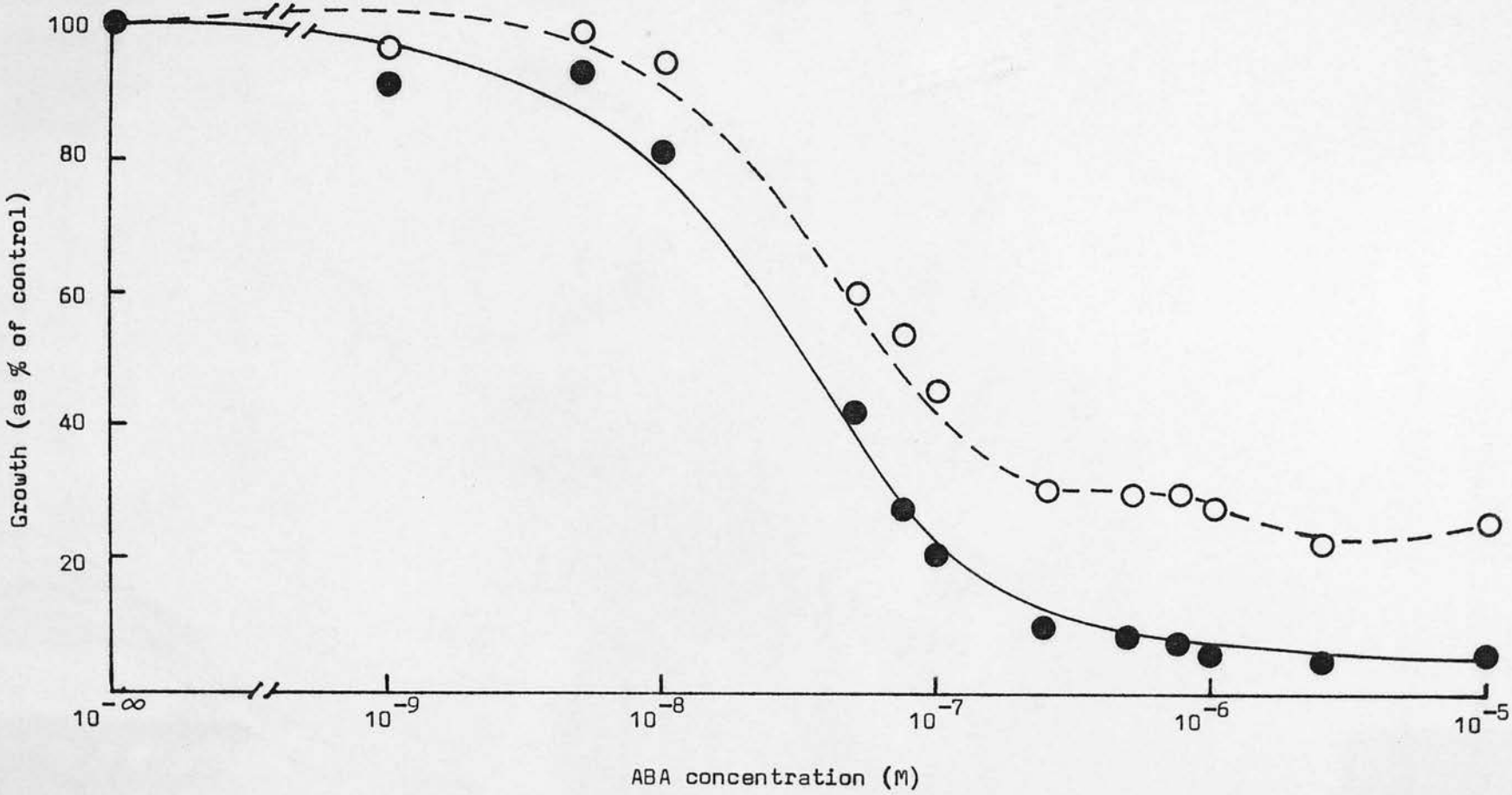

100
80
60
40
20
Growth (as % of control)
$10^{-\infty}$
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
ABA concentration (M)

FIGURE 6b


100
80
60
40
20
Growth (as % of control)
10^-∞
10^-9
10^-8
10^-7
10^-6
10^-5
ABA concentration (M)

above concentration ranges or in control cultures, even old overcrowded ones (cf. Czopek, 1959, 1963).

The production of turions in these clones of S. polyrrhiza must therefore rely on a threshold level of ABA. Above this level of ABA turions are produced abundantly, each clone having a particular concentration requirement for optimum turion production as shown in figure 7 (10^{-7} M ABA for S. polyrrhiza (N) and 5×10^{-7} M ABA for S. polyrrhiza (UC). However, above a certain concentration of ABA turion production ceases, possibly because initiation of the turion primordium requires a certain level of cell division and elongation, and higher levels of ABA inhibit growth to such an extent that these processes can no longer operate.

Stewart (1969) also found that growth inhibition by ABA was accompanied by turion formation in S. polyrrhiza after 5 days. It is interesting to note that even at $5{\cdot}68 \times 10^{-11}$ M ABA turions were produced with no concomitant growth inhibition, which possibly indicates that the strain used was particularly sensitive to ABA with respect to turion formation. Moreover he reported no turion formation in control cultures up to 18 days. At the same time Perry and Byrne (1969) reported the formation of turions in S. polyrrhiza within 10 days under environmentally non-inductive conditions (Perry, 1968) with ABA concentrations as low as 5×10^{-8} M.

The sensitivity of S. polyrrhiza to ABA obviously depends upon the strain used and probably also on growth conditions. Perry and Byrne (1969) found that strain 2-66 from South America did not produce turions at any concentration of ABA or indeed with any manipulation of light intensity, photoperiod, temperature or nitrate concentration (Perry, 1968). This strain also showed no growth

Figure 7

The effect of ABA on turion production in S. polyrrhiza. At certain concentrations of ABA, S. polyrrhiza produced turions. The number of turions produced was expressed as a % of the total number of new primordia formed after 8 days incubation in ABA.

● S. polyrrhiza (N)

○ S. polyrrhiza (UC)

The data for S. polyrrhiza (N) and (UC) are shown in comparison with other strains published in the literature which are indicated by authorship.

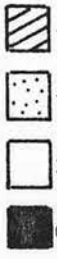
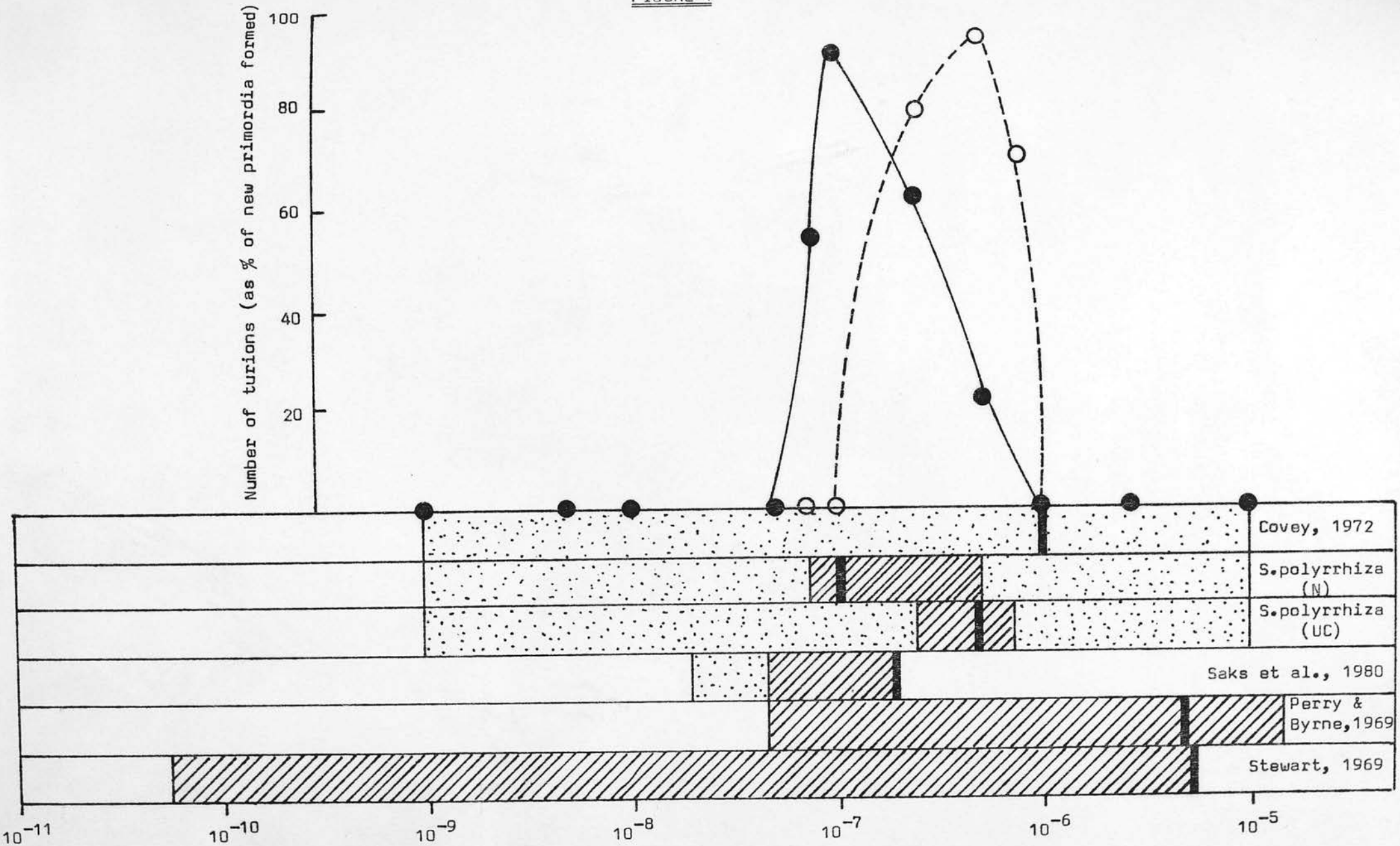


FIGURE 7


Number of turions (as % of new primordia formed)
100
80
60
40
20
Covey, 1972
S.polyrrhiza (N)
S.polyrrhiza (UC)
Saks et al., 1980
Perry & Byrne, 1969
Stewart, 1969
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}

inhibition with any concentration of ABA tested.

Although Stewart (1969) reported turion formation from $5{\cdot}68 \times 10^{-11}$ M to $5{\cdot}68 \times 10^{-6}$ M ABA with the maximum number of turions being produced at the highest concentration, it is not known if this was the optimum concentration for turion production, since no results were published for higher concentrations of ABA. Likewise Perry and Byrne (1969) reported turion formation with 5×10^{-8} to $1{\cdot}5 \times 10^{-5}$ M ABA, but optimum turion production was reached at 5×10^{-6} M. Perry and Byrne suspected that turions could be induced by lower concentrations of ABA if they were left up to 20 days, but their control cultures were by this time also forming turions. More recently, with a strain of S. polyrrhiza which did not so readily form turions under environmentally non-inductive conditions, Saks et al. (1980) showed that turion formation was induced by a threshold level of 5×10^{-8} M ABA, turion production being highest at 2×10^{-7} M. Unfortunately these authors also did not investigate the effect of higher concentrations of ABA on the rate of turion production.

The rate of turion production for the 2 clones used in this study is shown in figure 7. The range of ABA concentrations inducing turion formation is much narrower than the ranges reported in the literature as discussed above; having a clearly defined upper and lower limit. Only in one unpublished work (Covey, 1972) is a clearly defined range of ABA concentration for turion formation reported. It was found that ABA induced turion formation only at a concentration of 10^{-6} M in the strain of S. polyrrhiza used.

In response to ABA S. polyrrhiza (UC) was the slightly more sensitive clone with respect to growth inhibition, but less

sensitive with respect to turion formation. The ability of S. polyrrhiza (N) to form turions at low, specific concentrations of ABA, its unchanging growth pattern and the fact that no turion formation was ever seen in control cultures, led to the choice of S. polyrrhiza (N) for further experimental work, although some experiments were duplicated with the other clone.

3.2.2 Which Fronds Become Turions?

When a turion inducing concentration of ABA was added to a culture of S. polyrrhiza, not all of the developing fronds became turions. In order to follow the process of turion formation at the biochemical level, it was necessary to be able to define any frond as either a developing turion or a developing vegetative frond.

After 3 days exposure to ABA, developing turions can easily be recognised by the accumulation of anthocyanin pigment at their distal ends, but before this time they are visually indistinguishable from developing vegetative fronds. For this reason individual fronds were treated with ABA and their growth monitored every day by recording frond length. It was then possible to retrace the development of an individual frond back to the time of ABA addition. For any given exposure to ABA, any frond could then be characterised as a developing turion or vegetative frond, and then dissected from the mother for biochemical analysis.

The growth of S. polyrrhiza (UC) fronds was monitored by drawing and by photography. Examples of the effect of 5×10^{-7} M ABA on the growth of individual fronds are shown in figures 8, 10 and 11; for comparison the growth of an untreated frond is shown in figure 9. Data accumulated from many such examples showed that only

Figure 8

The growth of an individual frond of S. polyrrhiza (UC) in 5×10^{-7} M ABA. The frond was innoculated onto H/2 medium in a small petri-dish and supplemented with ABA. The frond was examined from below using an inverted microscope and drawn every day after ABA addition. The length of each frond was recorded with an eye-piece graticule and is shown on the figure in mm. Each frond was assigned a distinguishing letter. Shaded areas represent pigmented areas on the fronds. Totally shaded fronds are turions.

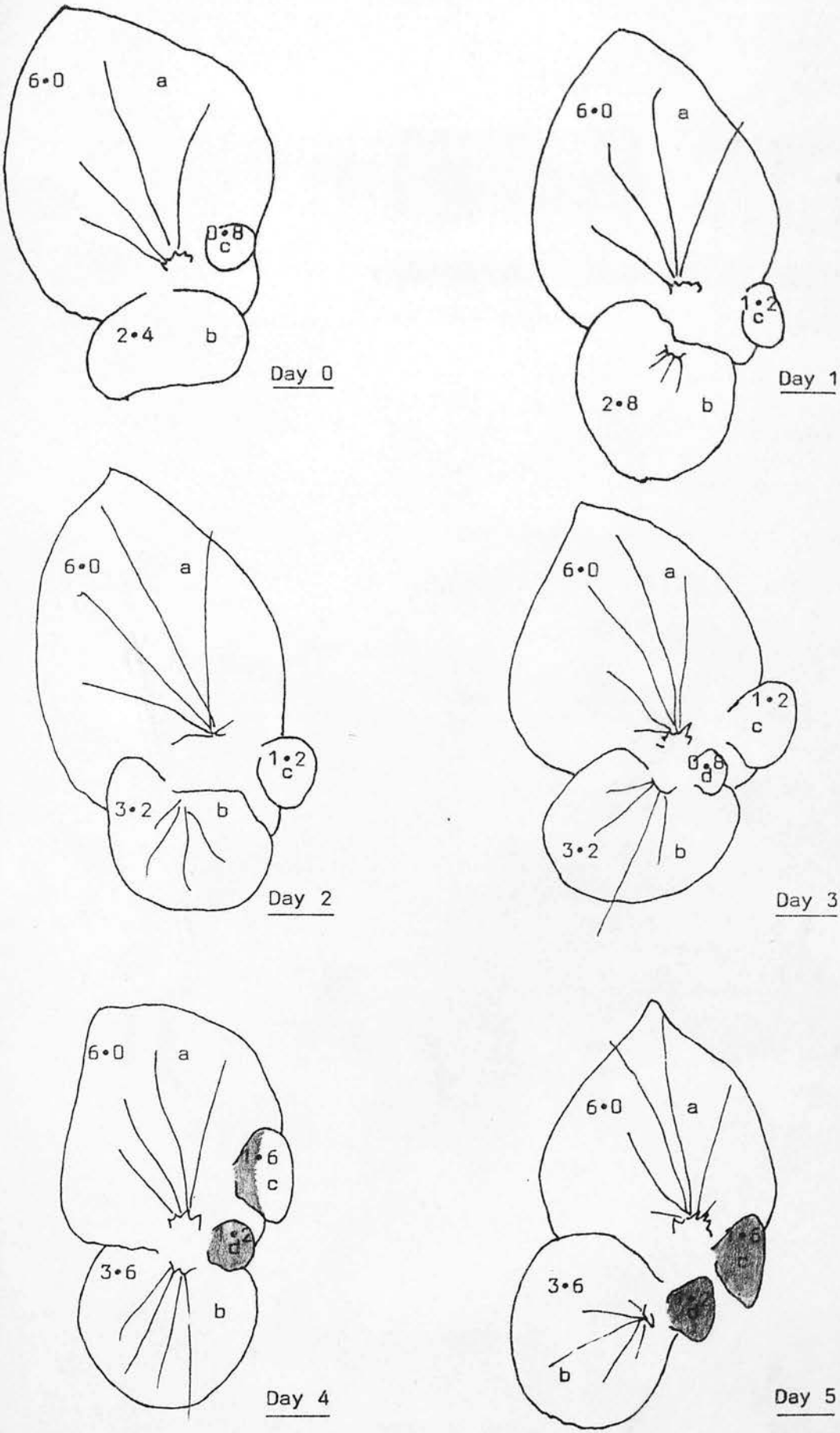

6•0
a
0•8
c
2•4
b
Day 0
6•0
a
1•2
c
2•8
b
Day 1
6•0
a
1•2
c
3•2
b
Day 2
6•0
a
1•2
c
0•8
d
3•2
b
Day 3
6•0
a
1•6
c
0•2
d
3•6
b
Day 4
6•0
a
1•6
c
3•6
b
Day 5

FIGURE 8

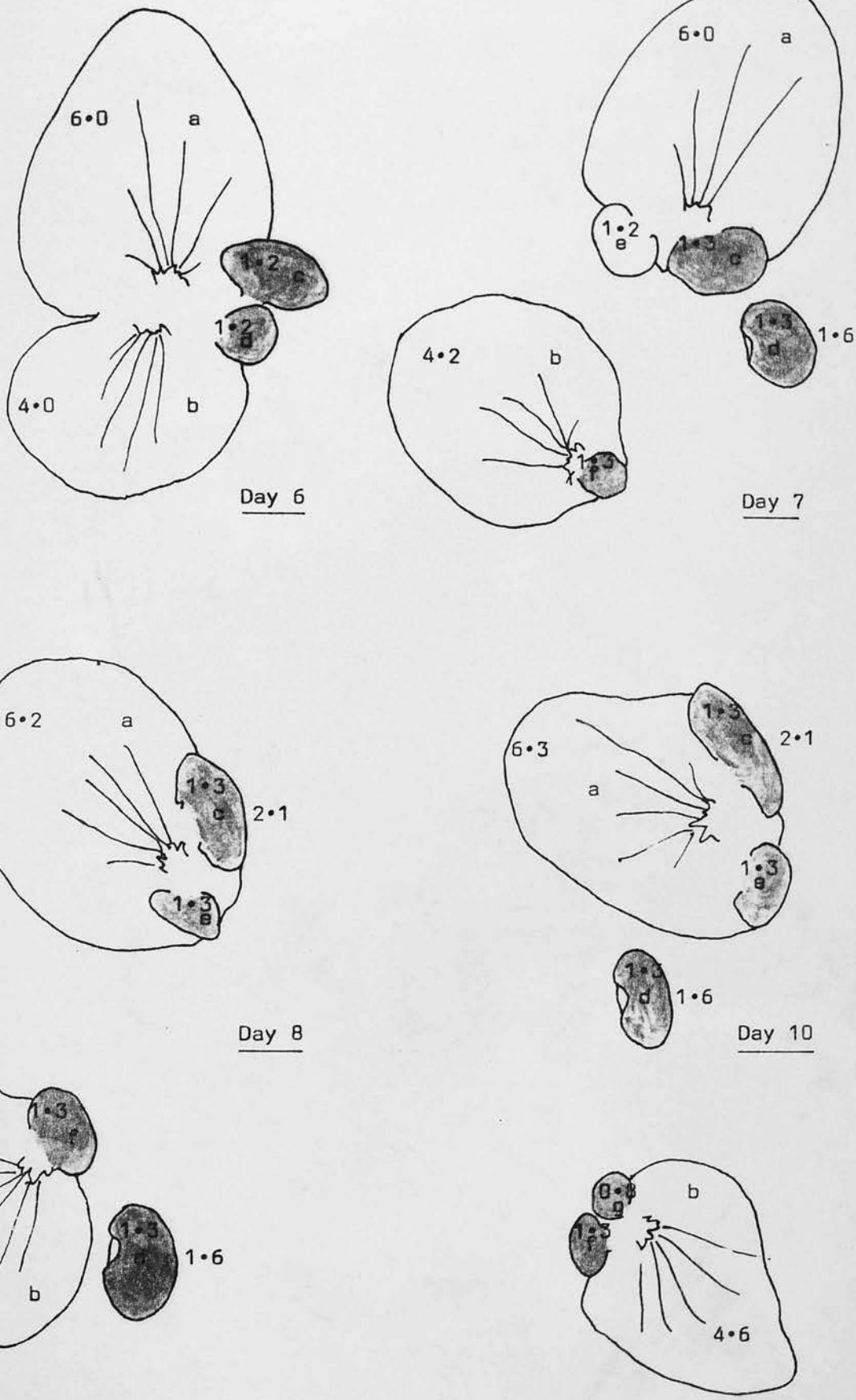


FIGURE 8 continued

Figure 9

The growth of an untreated individual frond of S. polyrrhiza (UC). The frond was innoculated onto H/2 medium in a petri-dish and photographed from below every day. Each frond was assigned a distinguishing letter and these, along with the length of the frond in mm are shown on the figure.

a) day 0

b) day 1

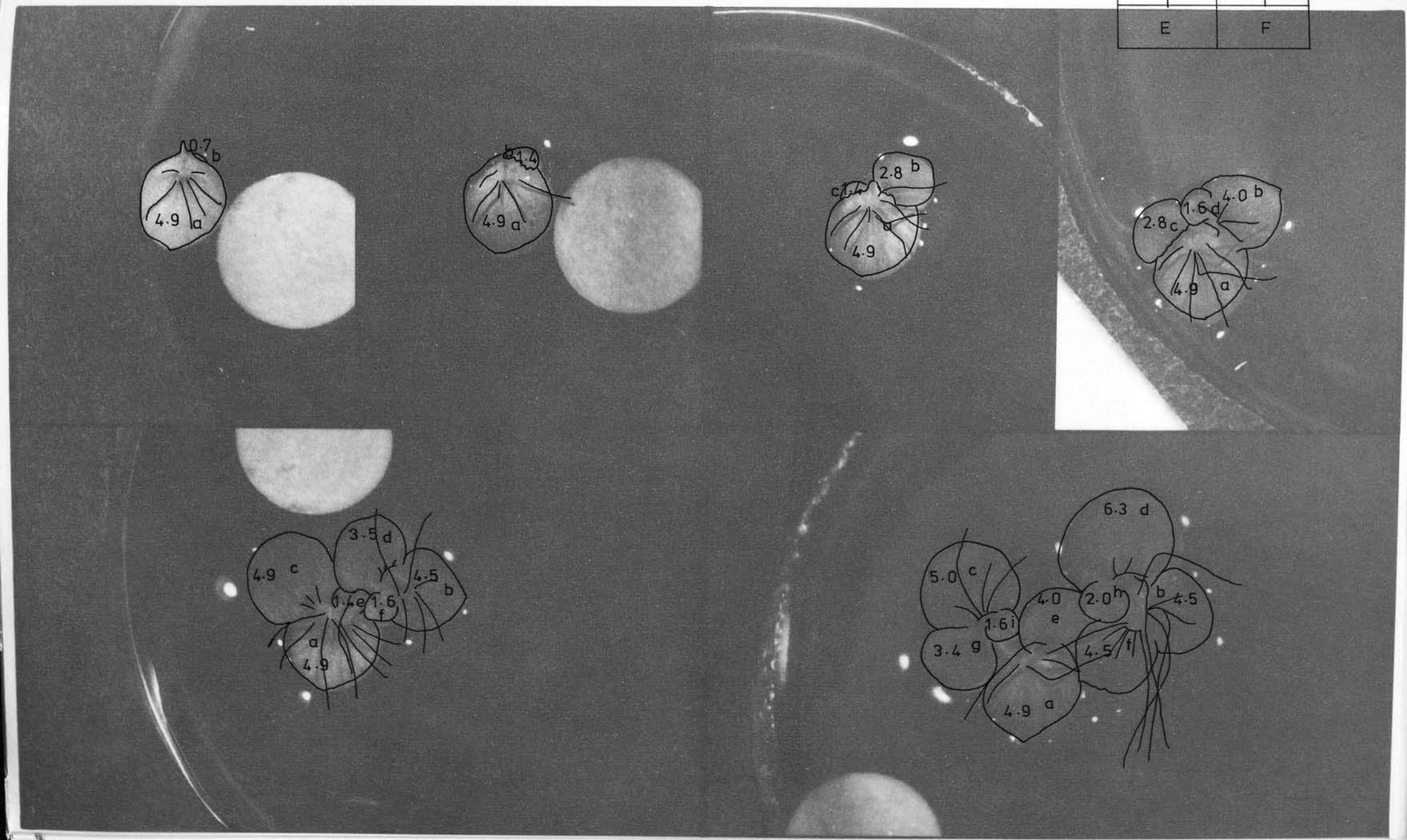
c) day 2

d) day 3

e) day 4

f) day 5

FIGURE 9


A
B
C
D
E
F
0.7 b
4.9 a
1.4 b
4.9 a
2.8 b
c
4.9
4.0 b
1.6 d
2.8 c
4.9 a
3.5 d
4.9 c
4.5 b
1.4 e
1.6 f
a
4.9
6.3 d
5.0 c
4.0 e
2.0 h
b 4.5
1.6 i
3.4 g
4.5 f
4.9 a

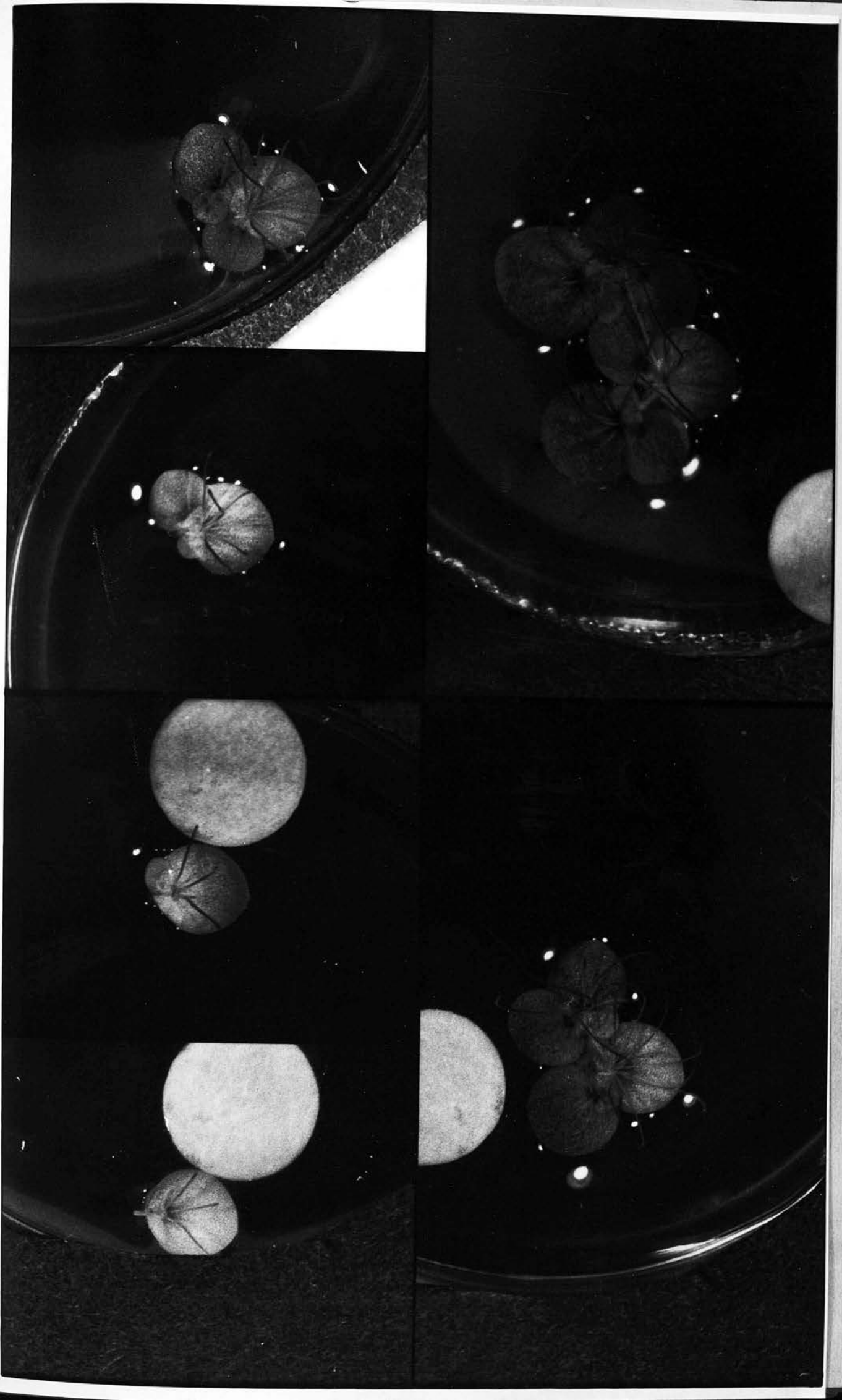


Figure 10 and Figure 11

Examples of the growth of individual fronds of S. polyrrhiza (UC), incubated in 5 x 10^{-7}M ABA. The fronds were innoculated onto H/2 medium containing ABA and photographed from below each day after ABA addition. Each frond was assigned a distinguishing letter which is shown along with the length of the frond in mm on the figures.

a) day 0

b) day 1

c) day 2

d) day 3

e) day 4

f) day 5

FIGURE 10

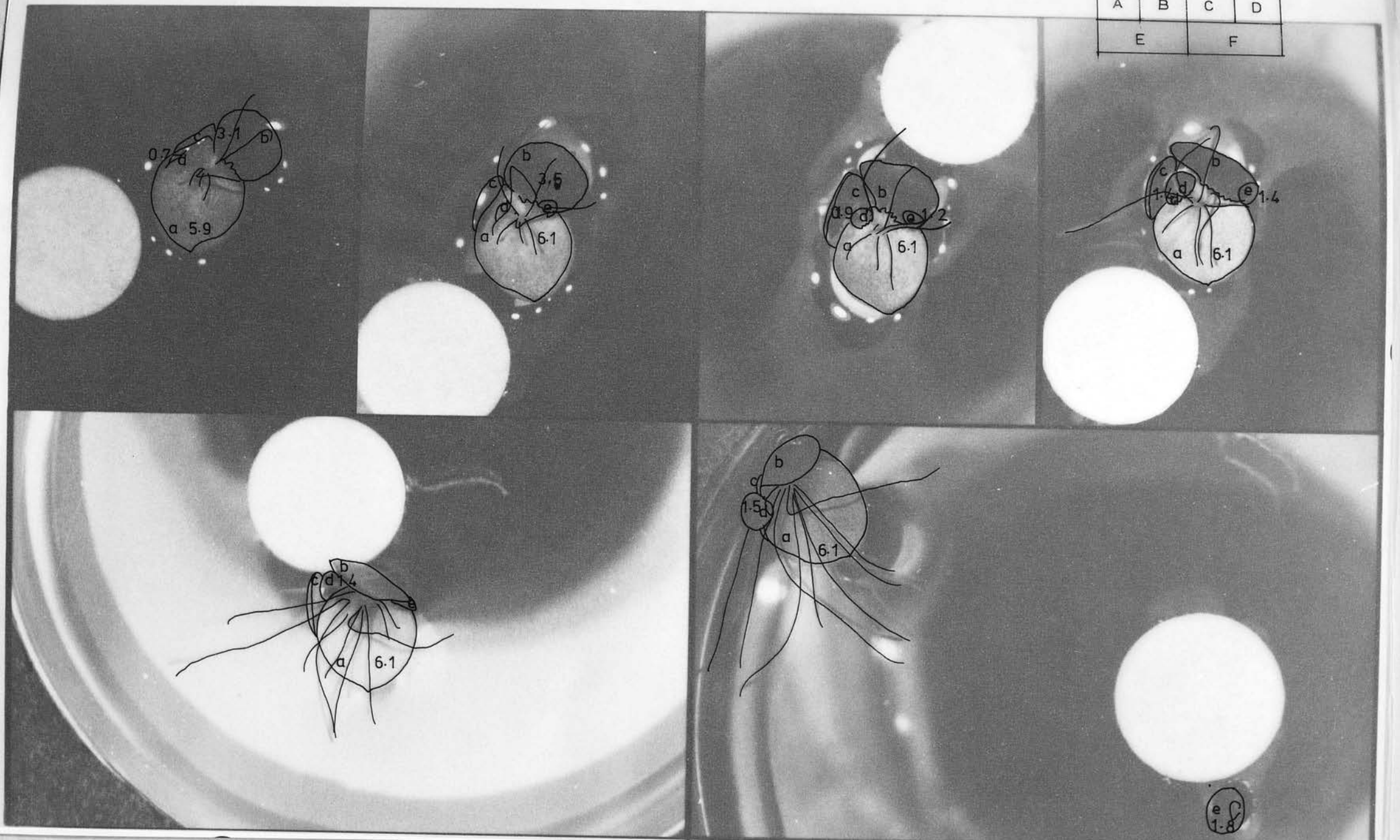
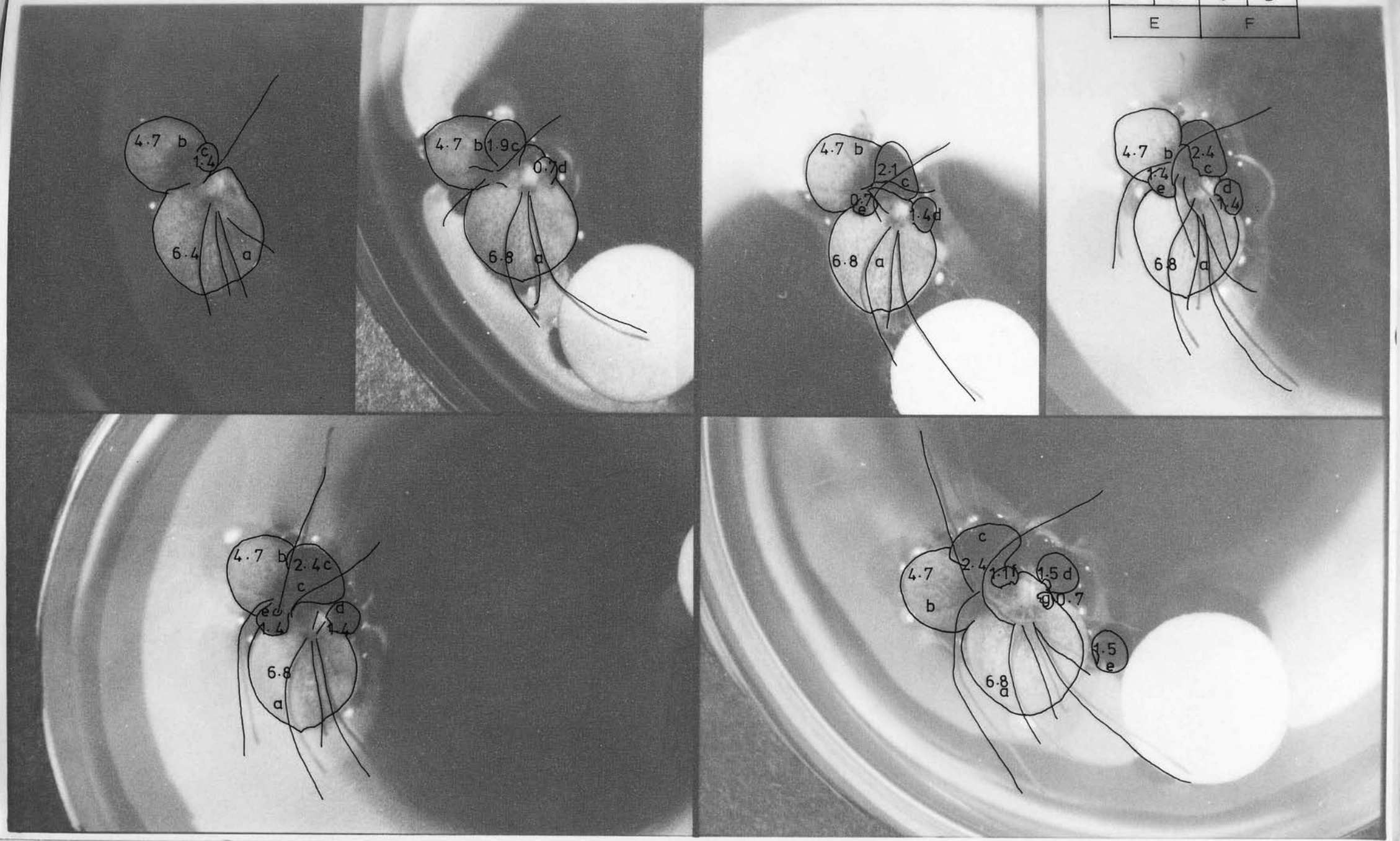

A
B
C
D
E
F
0.7
d
3.1
b
a 5.9
c
b
3.5
d
e
a
6.1
c
b
1.9
d
1.2
a
6.1
c
b
1.4
d
e
1.4
a
6.1
c
d 1.4
b
a
6.1
b
c
1.5
d
a
6.1
e
1.8

FIGURE 11


A
B
C
D
E
F
4.7 b
c 1.4
6.4 a
4.7 b 1.9 c
0.7 d
6.8 a
4.7 b
2.1 c
0.7 e
1.4 d
6.8 a
4.7 b
2.4 c
1.4 e
d 1.4
6.8 a
4.7 b
2.4 c
c
e 1.4
d 1.4
6.8 a
c
2.4
4.7 b
1.1
1.5 d
0.7
1.5 e
6.8 a

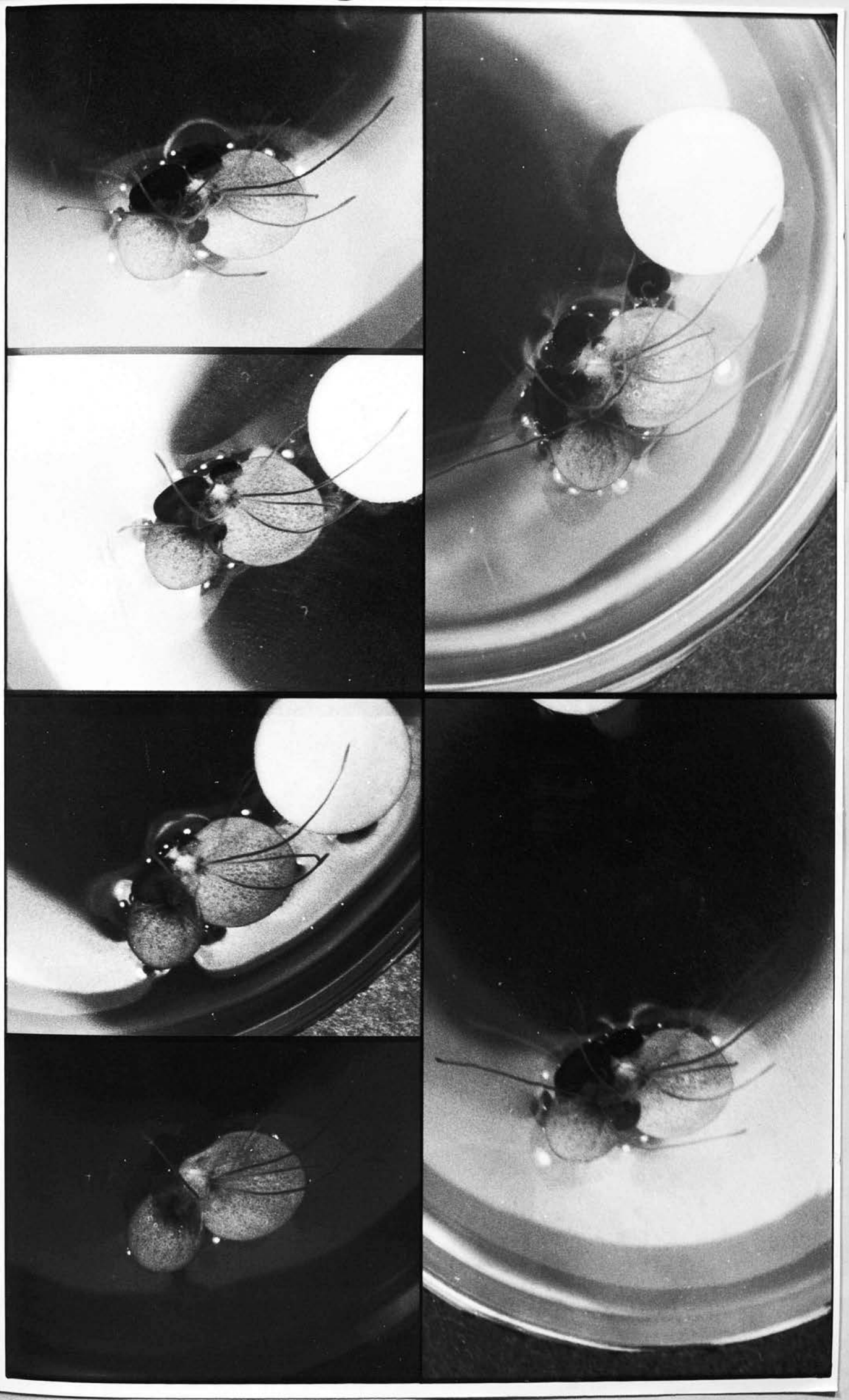


Table 1: Fronds destined to become turions

a) S. polyrrhiza (UC)

Time in ABA (days)	⩽ Length of frond (mm)
0	0•8
1	1•3
2	1•6
3	1•6
4	2•1
5	2•1
6	2•1
7	2•1
8	2•1
10	2•1

b) S. polyrrhiza (N)

Time in ABA (days)	⩽ Length of frond (mm)
0	0•7
1	1•0
2	1•5
3	1•8
4	1•8
5	1•8
6	2•0
7	2•0

Data collected from from all the figures such as Figs. 8 - 14 were collated to produce table 1. It shows for each day after addition of ABA, the length below which a frond must be for it to develop into a turion. a) 5×10^{-7}M ABA, b) 1×10^{-7}M ABA.

fronds 0•8 mm and below at the time of ABA addition developed into turions. Fronds longer than 0•8 mm but shorter than 1•3 mm developed into small vegetative fronds with a turion-like proximal end. These are referred to as semi-turions (Plate 1d, 3d). The formation of semi-turions indicates that the cells of the distal end of the developing frond had lost their sensitivity to ABA by the time of ABA addition, whereas the cells at the proximal end responded to ABA by accumulating anthocyanin, and differentiating into typical turion tissue (section 3.4.3). Semi-turions, although not so named, were also noted by Stewart (1969), but only at 5•68 x 10^{-6} M ABA. Although he found turion formation at much lower concentrations, he reported no semi-turions. His data are also consistent with a change in the sensitivity of the cells to ABA throughout the development of the primordium.

It is proposed that with low concentrations of ABA, only the particularly ABA sensitive cells within young primordia follow the turion developmental programme. Primordia well developed before the addition of ABA will be unaffected and grow into 'normal' vegetative fronds. With higher ABA concentrations the proximal cells of a primordium formed before ABA addition now respond to ABA because of its higher level within the cells.

Table 1a shows the length below which all fronds dissected out each day after ABA addition, would have developed into turions. Similar data were obtained for S. polyrrhiza (N) (Table 1b), and representative examples of the individual growth of fronds of this clone are shown in figures 12, 13 and 14. In this clone only fronds $\leqslant$ 0•7 mm in length at the time of ABA addition developed into turions.

Figure 12

The growth of an individual untreated frond of S. polyrrhiza (N). The frond was innoculated onto H/2 medium and photographed from below every day. Each frond was assigned a distinguishing letter and these, along with the length of the frond in mm are shown in the figure.

a) day 0

b) day 1

c) day 2

d) day 3

e) day 4

f) day 5

g) day 6

h) day 7

i) day 7 after having been separated

Figure 13 and Figure 14

The growth of individual fronds of S. polyrrhiza in 1×10^{-7} M ABA. The fronds were innoculated into H/2 medium supplemented with ABA and photographed from below each day after ABA addition. Each frond was assigned a distinguishing letter which is shown along with the length of the frond in the figures.

a) day 0

b) day 1

c) day 2

d) day 3

e) day 4

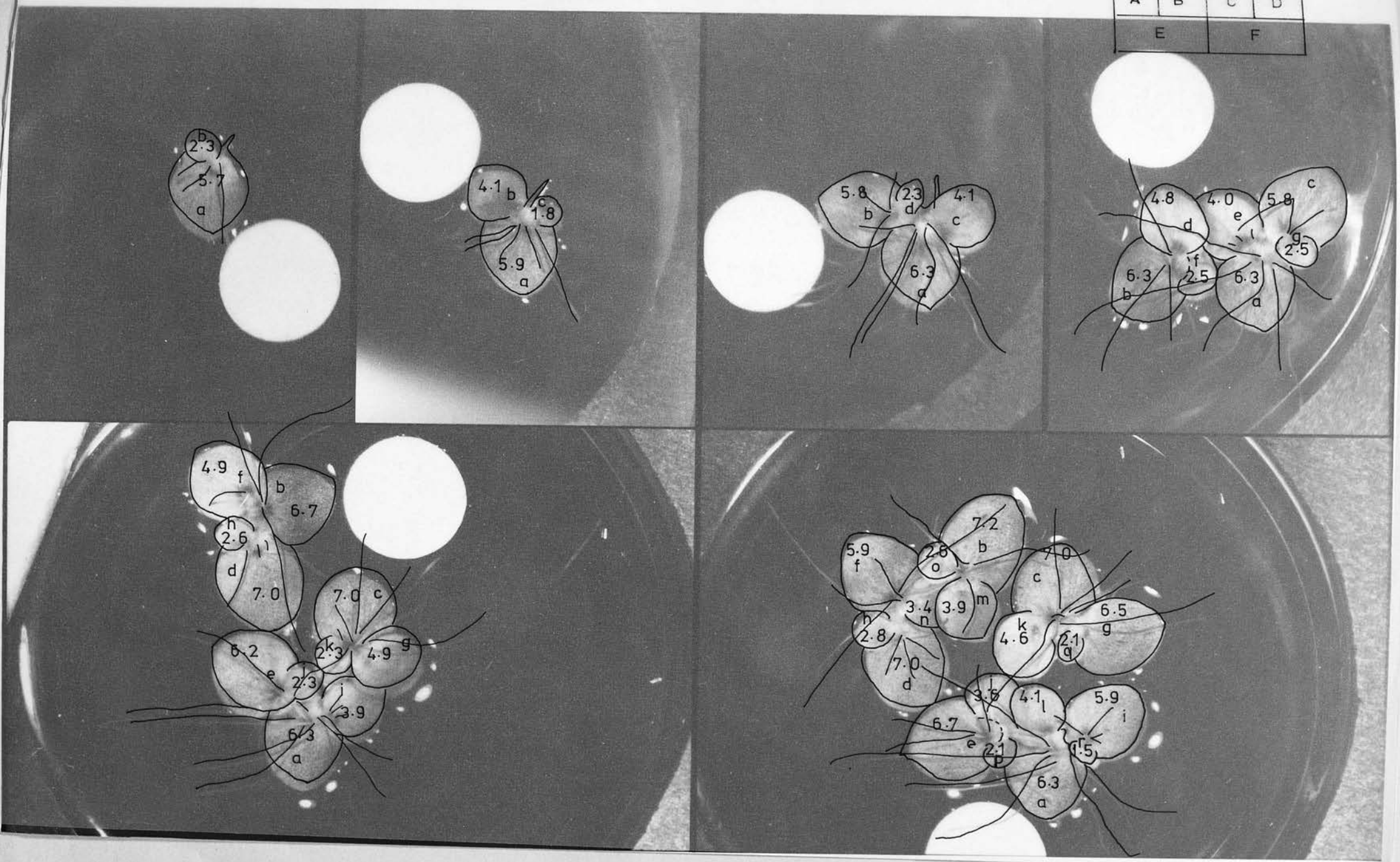
f) day 5

g) day 6

h) day 7

i) day 7 after having been separated

FIGURE 12


A
B
C
D
E
F
2·3
5·7
a
4·1
b
c
1·8
5·9
a
5·8
b
2·3
d
4·1
c
6·3
a
4·8
d
4·0
e
5·8
c
f
g
2·5
6·3
b
2·5
6·3
a
4·9
f
b
6·7
h
2·6
i
d
7·0
7·0
c
6·2
e
k
2·3
4·9
g
2·3
j
3·9
6·3
a
7·2
b
5·9
f
2·8
o
7·0
c
3·4
n
3·9
m
6·5
g
h
2·8
4·6
k
2·1
q
7·0
d
3·6
l
4·1
l
5·9
i
6·7
e
2·1
p
r
5·5
6·3
a

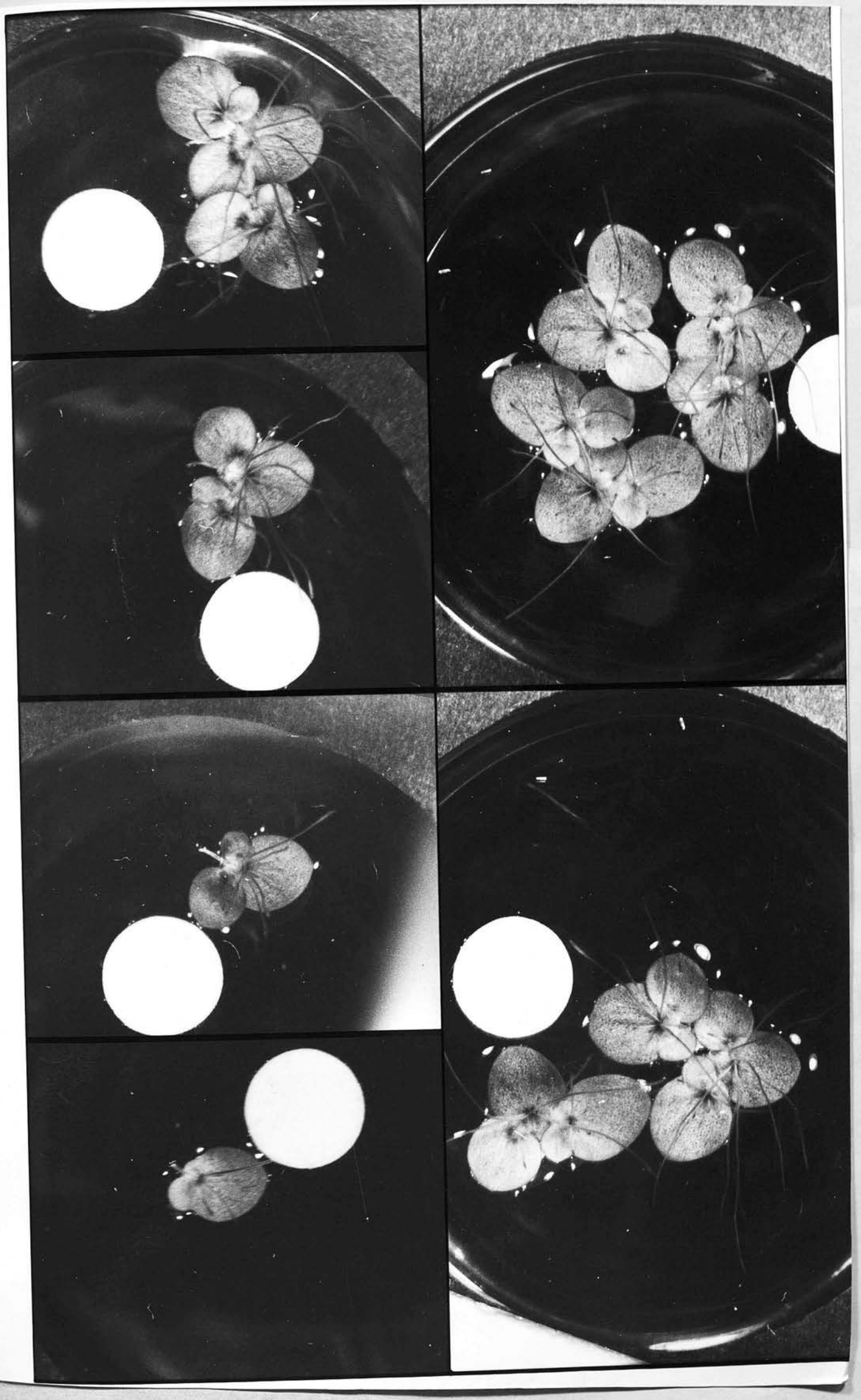


FIGURE 12 continued

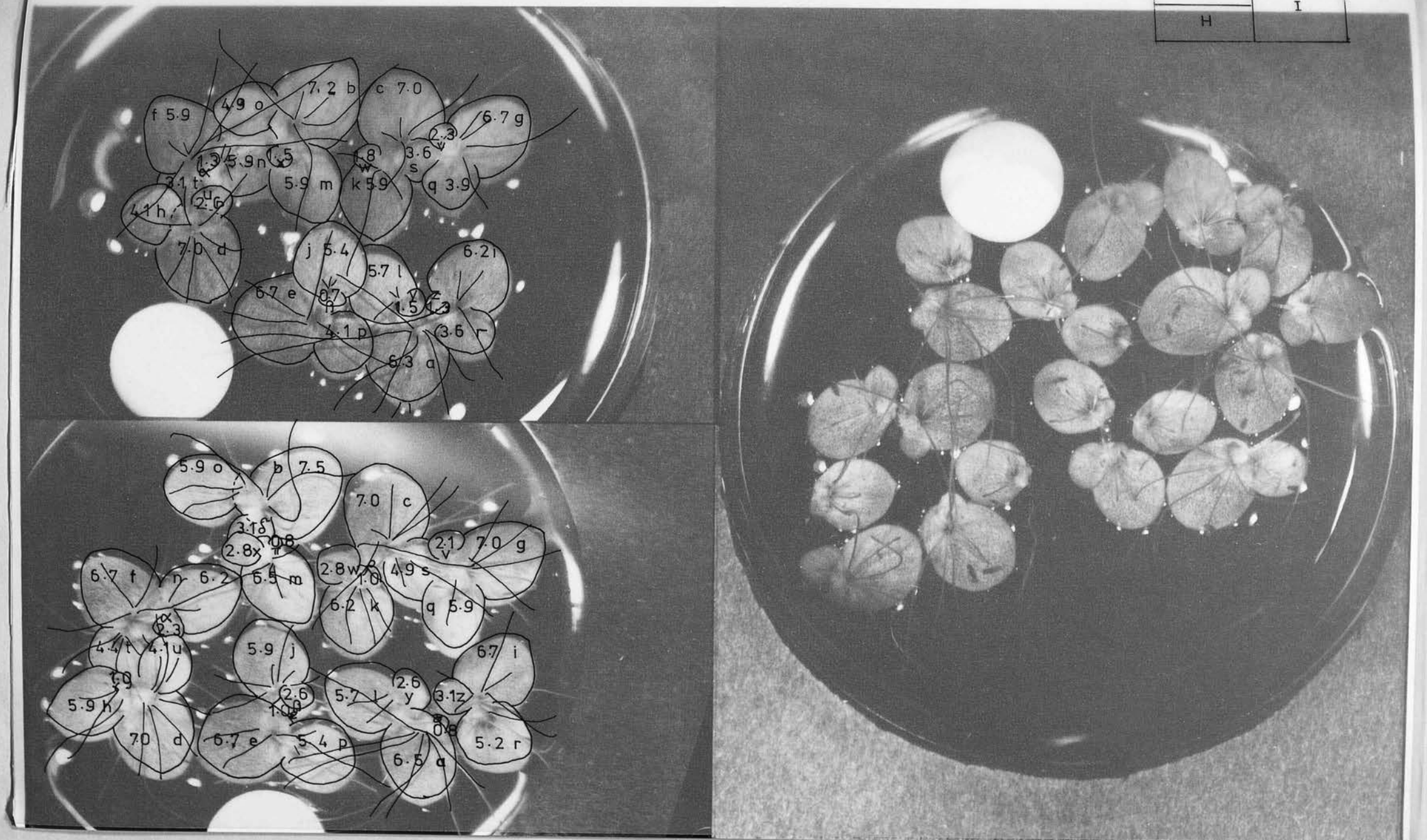
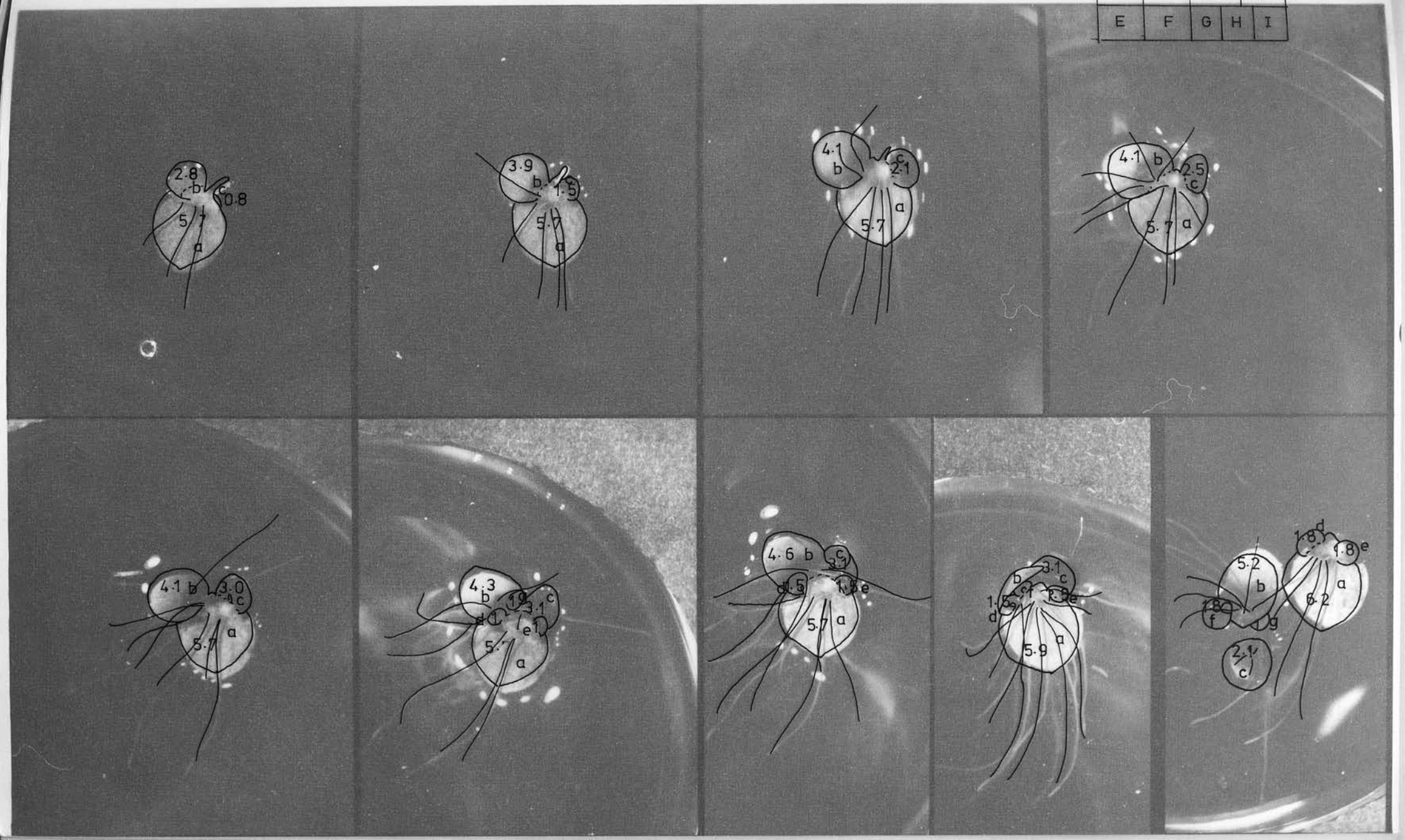


FIGURE 13


A
B
C
D
E
F
G
H
I
2.8
b
c
0.8
5.7
a
3.9
b
1.5
5.7
a
4.1
b
c
2.1
5.7
a
4.1
b
2.5
c
5.7
a
4.1
b
3.0
c
5.7
a
4.3
b
1.9
c
3.1
d
e
5.7
a
4.6
b
c
3.1
d
1.5
e
5.7
a
3.1
b
c
1.5
d
e
5.9
a
1.8
d
1.8
e
5.2
b
a
6.2
f
g
2.1
c

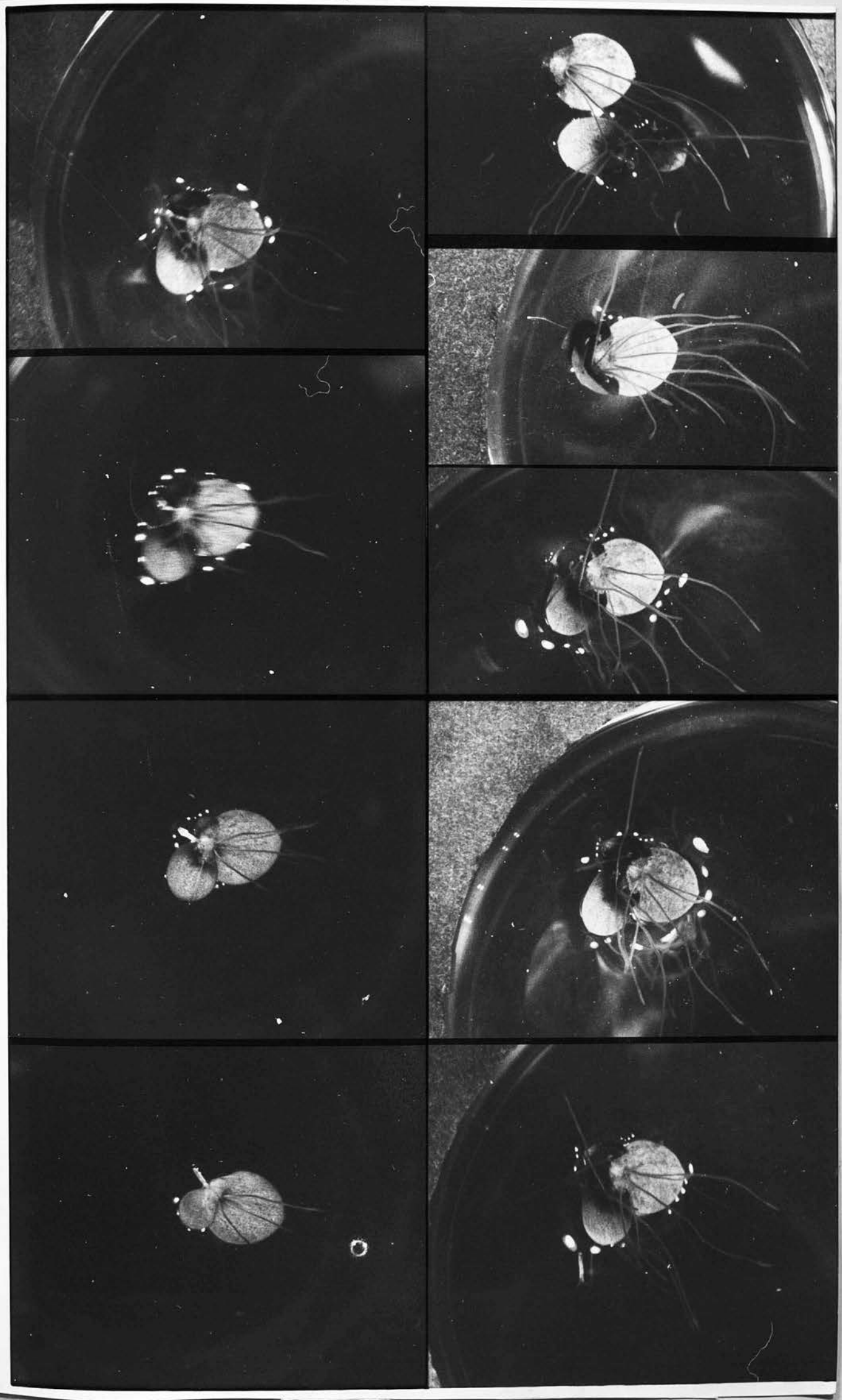
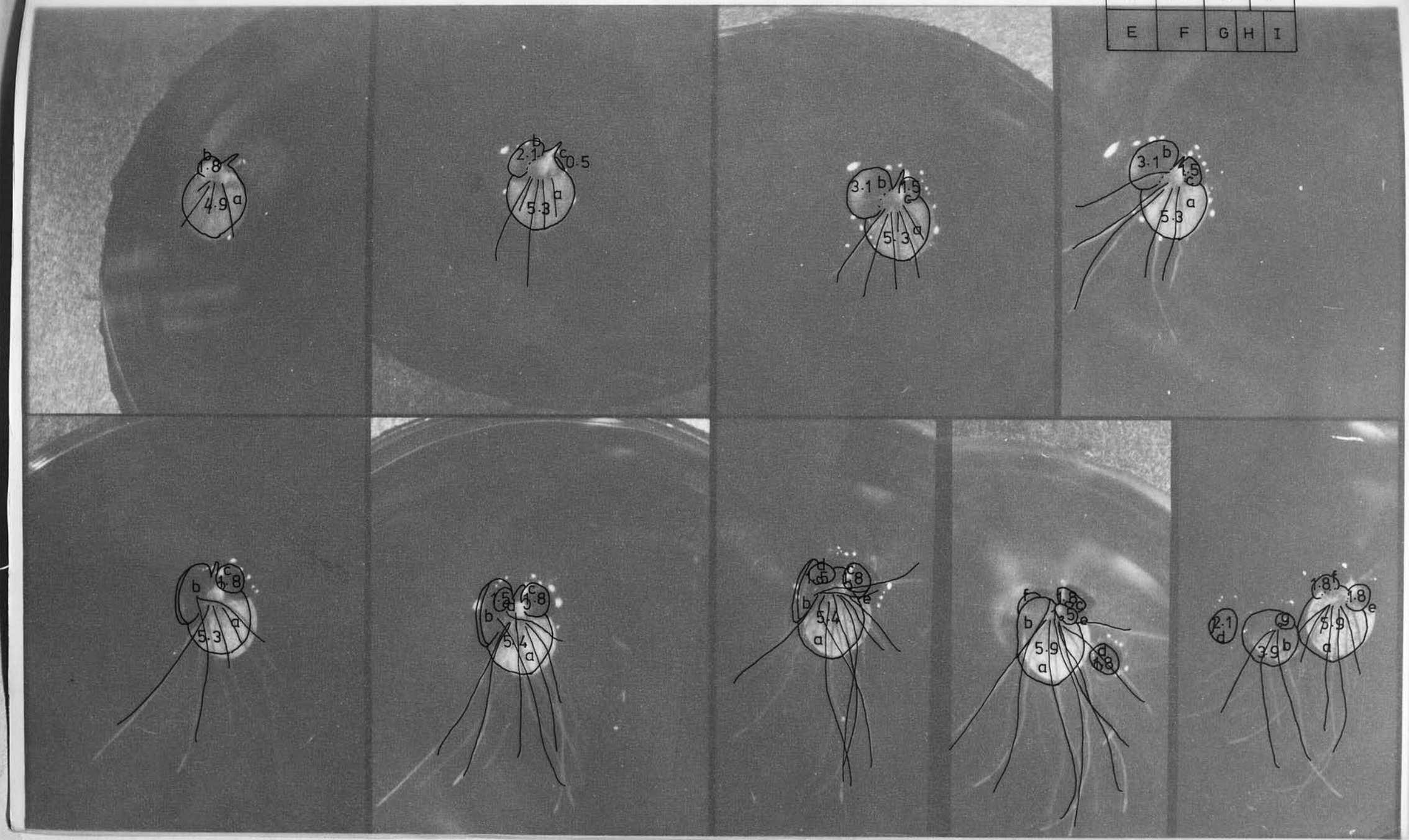
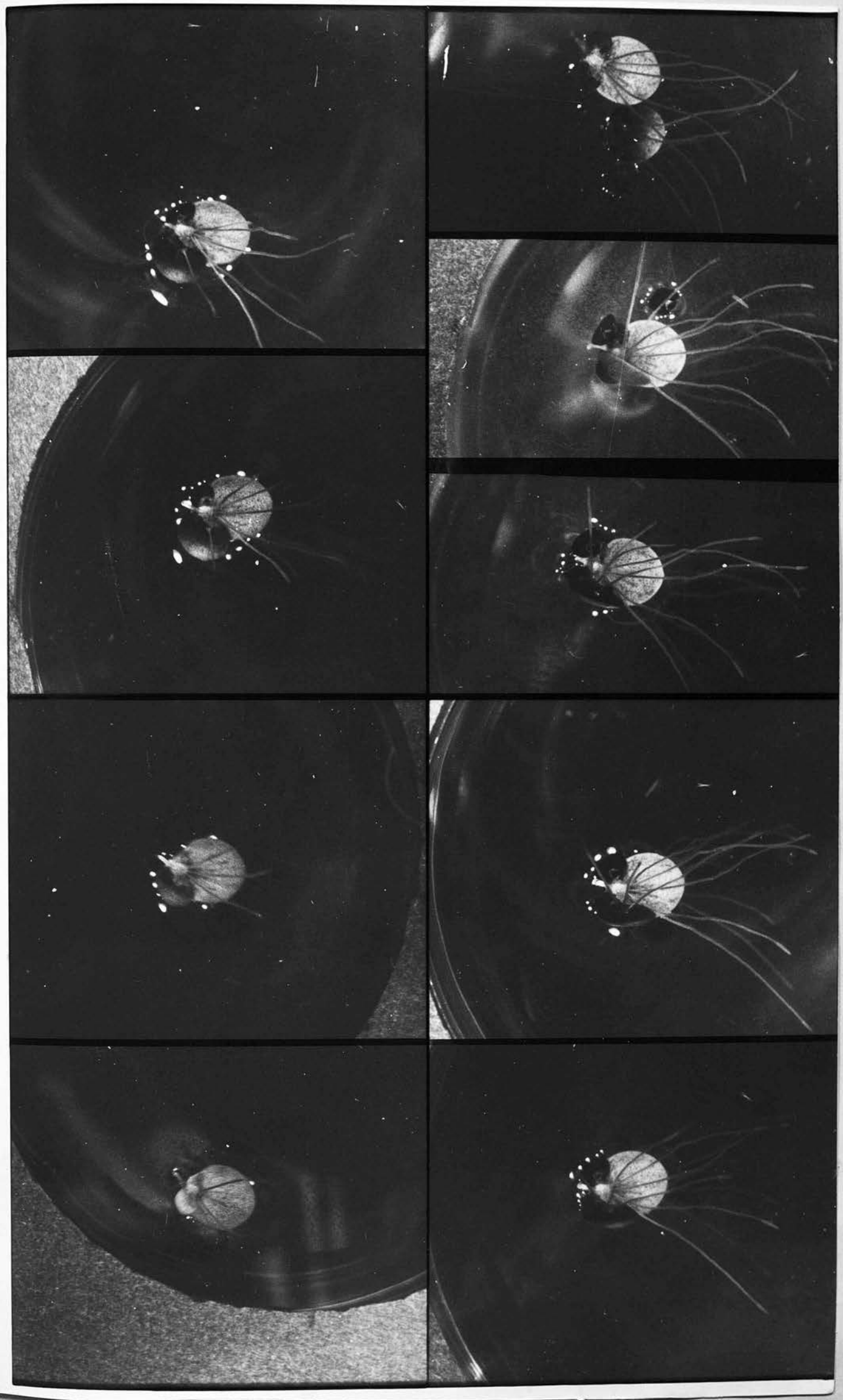


FIGURE 14


A
B
C
D
E
F
G
H
I
b
1.8
4.9
a
b
2.1
c
0.5
5.3
a
3.1
b
1.5
c
5.3
a
3.1
b
1.5
c
a
5.3
b
c
1.8
5.3
a
1.5
c
1.8
b
5.4
a
d
1.5
c
1.8
b
e
5.4
a
f
1.8
c
5
e
b
5.9
a
d
1.8
2.1
d
9
3.9
b
f
1.8
1.8
e
5.9
a



3.2.3 Is Turion Formation Reversible?

The time course and reversibility of the promoted changes by ABA were followed by incubating S. polyrrhiza (N) fronds in 1×10^{-7} M ABA and transferring them, at intervals, to medium free of ABA.

Turions were only formed by plantlets which had been incubated for 5 days or more. Turions still attached to the mother frond when ABA was removed, germinated rapidly without first sinking. This indicated that a continual supply of ABA was necessary for the turion to fully mature, abscise, sink and remain dormant. Up to 5 days in ABA, the process of turion formation was reversible. However, although no normal turions were produced in less than 5 days exposure, morphological changes in the fronds produced in the presence of shorter exposures to ABA were observed.

Plantlets incubated in ABA for 6, 12, 24 and 48 hours and then transferred to ABA free medium did not produce turions at up to 7 days after ABA addition and appeared identical to control plantlets (Plate 1a). Only after 72 hours in ABA were there visible signs that the processes involved in turion formation had begun. Plate 1b shows a daughter frond which has a heavily pigmented rim of tissue at its distal end. This pigmentation is characteristic of the turion and is never seen in control fronds. At the time of ABA addition, this frond would have been a tiny primordium deep within the mother pocket, and would have developed under the influence of ABA for 3 days. The cells of the distal end of the young frond were sensitive to ABA and responded by developing into turion-type cells. The cells of the younger proximal portion of the frond, although possibly sensitive to ABA at some stage, did not develop under the

direction of ABA for long enough for morphological changes to take place within the tissue.

The reversible nature of the processes leading to turion formation up to 3 days in ABA indicates that many sequences of biochemical events are necessary before a cell can develop into a turion-type cell. Only when fronds have been incubated for 5 days in ABA do all the cells develop into turion types and typical turions emerge (Plate 1c). These turions are pigmented at the proximal as well as the distal end, and have the reniform shape and large pocket sheath so characteristic of the fully mature turion. If ABA is removed after this time the cells of the proximal end cannot now switch to vegetative frond cells, since they have completed their development. The turion does respond to removal of ABA however by germinating within 2 days of abscission, producing a vegetative frond from one of its meristematic pockets.

This finding is in contrast to the situation found by Stewart (1969). He found that only 24 hours in ABA were necessary to initiate the biochemical events which switch the developmental pattern of the primordia from vegetative fronds to turions, this sequence of events being readily reversible. However, he did note that the first fronds formed after transfer into ABA free medium were "intermediate in appearance between the turion and the normal vegetative frond". Unfortunately no reference was made as to whether fully mature turions were formed after only 24 hours in ABA i.e. what exposure of ABA was necessary to produce a fully developed turion.

3.2.4 Turion Germination

ABA induced turions from both clones of S. polyrrhiza abscised from the mother frond after approximately 7 days and sank to the bottom of the flask. If they were then left in the flask, they remained dormant for up to 3 months. If however, they were transferred to ABA free medium, they buoyed to the surface after 24 hours and germinated within 4 days. They possessed no vernalisation requirement as reported for turions induced by manipulations of temperature, photoperiod and light intensity (Jacobs, 1947; Perry, 1968) and those induced by sucrose (Czopek, 1959, 1963; Newton et al., 1978). Sibaski and Oda (1979) reported that S. polyrrhiza turions formed in response to nitrogen deficiency germinated readily without pre-treatment with low temperature. In accordance with Jacobs' findings, germinating turions of S. polyrrhiza were found to give rise to vegetative fronds only and never to turions.

3.3 ORGANISATION OF THE VEGETATIVE FROND

A complete study of the organisation of the vegetative frond was felt to be necessary before its development was investigated.

3.3.1 Gross Morphology

The mature frond of S. polyrrhiza is broadly ovate, slightly asymmetric in shape and has approximately 7 veins running the length of the frond (Plate 1a, 2a). The frond commonly reaches a length of 6 mm and a width of 5 mm, although fronds as large as 8 mm x 6 mm have been observed. The dorsal surface of the frond is bright green, glossy and water-repellent (Plate 3a), whereas the ventral surface is duller and often pigmented with anthocyanin, especially

in the actively growing regions (Plate 3b).

The mother frond produces a daughter frond from each of 2 meristematic pockets near to its proximal end, usually the left hand pocket producing the first daughter in S. polyrrhiza (N) and the right hand pocket in S. polyrrhiza (UC), when the mother frond is viewed from its dorsal proximal end (see Figs. 12 and 9).

The meristematic pockets can be seen more clearly on the ventral surface (Plate 2b). The pocket sheath covering the meristematic regions is often slightly larger on the side producing the first daughter. Just below the pockets is the node (cf. nodal embryonic point, Rimon, 1964; centrum, Jacobs, 1947) which is the point of attachment of the roots (Plate 3b). The roots are regarded as adventitious (Hillman, 1961) and form at the node just above the lower epidermis. S. polyrrhiza commonly produces 5 - 7 roots from each frond.

3.3.2 Anatomy

A transverse section of the vegetative frond (Plate 4a) reveals the cell types that compose the three basic tissues: epidermal, mesophyll and vascular tissue. Both the upper and lower epidermis consist of a single layer of cells.

3.3.2.1 Upper epidermis

Cells of the upper epidermis contain a thin layer of cytoplasm in which an occasional chloroplast is found (Plate 4b), and are often pigmented with anthocyanin. This finding is in contrast to the light microscope study by Jacobs (1947) who reported that "the upper epidermis consists of small cells that lack contents". Le Pabic (1972) confirms Jacobs' work in his ultrastructural

analysis of the chloroplasts of S. polyrrhiza. However, when working at high magnifications, rare components such as epidermal cell chloroplasts are likely to be missed. In plate 5 the structure of the upper epidermal cell can be more clearly seen. The cells have a thin layer of cytoplasm surrounding a large central vacuole. The cytoplasm contains chloroplasts and all the typical higher plant cell organelles including the nucleus.

Epidermal cells of Wolffia arrhiza (Anderson et al., 1973) and Lemna minor (Melaragno, 1974) were also found to contain chloroplasts. Anderson and co-workers point out that although chloroplasts are generally absent from epidermal layers in plants (Esau, 1965), few detailed studies on the fine structure of plant epidermal cells are available. Thus a real need exists for information at the ultrastructural level with regard to epidermal cells in duckweeds.

Stomata are also present in the upper epidermis and their guard cells appear to contain many chloroplasts (Plate 7a). This observation is confirmed by Jacobs (1947) and later by Le Pabic (1972). In the paper by Anderson et al. (1973) an electron micrograph of a guard cell of Wolffia arrhiza is shown, which also contains a relatively dense cytoplasm. Under the electron microscope the guard cell of S. polyrrhiza has a very dense cytoplasm with fairly large vacuoles (Plate 19d). Although the chloroplasts are not seen in this section, numerous mitochondria and ribosomes are present, along with endoplasmic reticulum and dictyosomes. The guard cell has a heavily thickened cell wall especially near the stomatal aperture, where it is elongated to form

a spine. This structure is very similar to that shown by S. oligorrhiza, a related species having only 1 root per frond like Lemna minor (Ledbetter and Porter, 1970). Lemna minor also has these rather unusual guard cells (Haberlandt, 1914), but no cellular contents were observed within the cell by Melaragno (1974).

Melaragno suggests that since the guard cells of most angiosperms contain a relatively dense cytoplasm, presumably to provide the metabolic machinery to regulate turgor within the cell and consequently the size of the stomatal aperture (Meidner and Mansfield, 1968), then Lemna minor may need no such machinery since the stomata remain open all the time (Wagner, 1973). In most plants it is important that the stomata close whenever appropriate in order to prevent excess water loss via transpiration. However, the problem of water loss leading to desiccation is probably of little consequence in the case of the Lemnaceae. Although there is no experimental evidence for stomatal movements in the Lemnaceae, Melaragno suggests that if small alterations in the stomatal aperture were necessary, this could be achieved in Lemna minor by changes within adjacent epidermal cells which would affect the position or inclination of the anticlinal walls of the guard cells.

If the guard cells of Lemna minor have no contents, then this appears to be the exception to the rule, and there is no report of mature stomata in any duckweed being able to regulate their apertures, whether the guard cells lack contents or not. Godziemba-Czyz (1969b) reports that whereas the stomata of the floating form of Wolffia arrhiza are widely open, the stomata of the submerged

form and of the turions are only slightly open. Melaragno takes this as evidence that Wolffia does regulate its stomatal aperture, but it should be noted that the floating and submerged fronds have formed their respective stomatal structures during their development. A floating frond can only produce a submerged frond from its reproductive pocket, and does not simply convert from one form to the other.

A similar situation was found in S. polyrrhiza. The stomata of the vegetative frond are widely open (Plate 6c), whereas the turion has fully closed stomata (Plate 6d). This finding confirms Jacobs (1947) who stated that "the stomata of the turion appear to remain closed until after germination", although this implies that the stomata opened at germination. Germination of the turion has not been studied in this work, but abscisic acid, which effects stomatal closure in many species (Mittelheuser and van Steveninck, 1969; Horton, 1971) had no effect on the stomatal aperture of the vegetative frond of S. polyrrhiza (N) even with 10 days exposure to 10^{-7} M ABA (Plate 7).

The lower surface of the guard cells, as well as portions of those of the adjacent epidermal cells are exposed to the sub-stomatal cavity.

3.3.2.2 Lower epidermis

Unlike the upper epidermis which is well cutinised, the lower epidermis has only a thin cuticle. Cells of the lower epidermis appear to be similar in structure, although slightly larger than the upper epidermal cells (Plate 4a). Many of the lower epidermal cells

are in direct contact with the large intercellular air spaces which dominate the lower half of the mesophyll. This finding is also in contrast to the anatomy found in Lemna minor (Melaragno, 1974), where none of the lower epidermal cells were ever found directly exposed to an air space.

A surface view shows the cells of the lower epidermis to be far more irregular in shape than those of the upper epidermis, and their outlines have a wavy appearance (Plate 4c). Under the electron microscope the cells have a large central vacuole limited by a thin layer of cytoplasm (Plate 8a). The chloroplasts contain starch and have well developed granal systems (Plate 8b).

3.3.2.3 Mesophyll

The main part of the mature mother frond is composed of aerenchyma i.e. parenchyma cells separated by large intercellular air spaces. This anatomy is an obvious structural adaptation to the habitat of the plant, since a relatively large volume of gas is maintained within the plant body adding considerably to the buoyancy of the plant.

The mesophyll appears to be differentiated into 2 main compartments; the upper half, referred to as palisade mesophyll (Jacobs, 1947), consists of more tightly packed rows of cells than those of the lower half, or spongy mesophyll. The palisade mesophyll varies between 2 - 6 cells deep, depending on the thickness of the frond (Plates 4a and 4d). Chloroplasts are abundant in the palisade and tend to lie on the side and bottom walls of the cells.

The spongy mesophyll consists of more irregularly spaced cells

which often form tiers delimiting the large intercellular air spaces so characteristic of the tissue (Plates 4a, 4c). There appears to be fewer chloroplasts in the cells of the spongy mesophyll. The parenchyma cells of the frond are arranged so that a major portion of their surface is exposed to intercellular air space. This anatomical feature not only lends structural support to the extensive air space network, but allows for the movement of gases directly into and out of the cells.

Ultrastructural analysis reveals that the cells of the mesophyll are much larger than the epidermal cells, but that the internal structure is very similar (Plate 8c). The chloroplasts tend to be elongated, although sometimes they are more circular (Plate 8d). The fine structure of the mature chloroplast is shown in plate 9a. The chloroplast is limited by a double membrane (the chloroplast envelope) and inside can be seen the thylakoid network consisting of stromal and granal lamellae. The grana rarely consist of more than 8 stacked lamellae. Osmiophilic globules (plasto-globuli) are often seen in the chloroplasts and starch grains are common (Plate 9b). The cells have a nucleus, numerous mitochondria (Plate 9c) and a large central vacuole which is granular in appearance (Plate 9d).

Scattered in the mesophyll, but especially in the palisade are cells, devoid of cytoplasmic contents, which contain various inclusions. The most common inclusions are needle-like raphides, presumably calcium oxalate crystals (Plates 10a, 10b). The other inclusions which are found, especially in developing fronds, are deposits of darkly staining material (Plate 10c). These cells have

been called subepidermal idioblasts (Jacobs, 1947) since they tend to be concentrated just below the upper epidermis of the vegetative frond (Plate 10d). Idioblasts contain flavin-like compounds (Witzum, 1974a) and may provide a protective function (Esau, 1965).

3.3.2.4 Vascular tissue

The vascular strands are simple unbranched veins radiating from the node and vary from 5 - 7 in number. They usually lie near the junction of the palisade and spongy mesophyll and are surrounded by a poorly defined sheath of parenchyma. Jacobs (1947) describes the orientation of the vascular tissue as "inverted with the xylem vessels on the bottom and the phloem elements above". Melaragno (1974) found the opposite arrangement in Lemna minor with each bundle consisting of a single tracheary element, 2 or 3 sieve elements and their related companion cells and from 5 to 10 small vascular parenchyma cells.

In S. polyrrhiza most of the vascular strands consist of 1 or 2 bundles, but the organisation of each bundle is difficult to discern at the light microscopic level (Plates 11a, 11b). However with the help of longitudinal sections (Plates 11c, 11d), where the spiral thickenings of the xylem vessel are more clearly seen, I conclude that the xylem is located dorsally and the phloem ventrally. Each bundle consists of a single tracheary element surrounded by 6 to 8 large parenchyma cells. Below are 1 or 2 sieve elements with their related companion cells and phloem parenchyma cells. Confusion may have arisen about the orientation of the vascular tissue because the xylem elements do not possess thickened cell walls. They do have annular or spiral thickenings but these are not seen in transverse

sections. The sieve tubes however do have relatively thick cell walls and could be mistaken for xylem vessels. The vascular bundle is separated from the large parenchyma cells or intercellular air spaces of the mesophyll by smaller vascular parenchyma cells.

The veins converge at the node from where a large bundle runs toward the proximal end forming the stolon. The stolon attaches the young frond to its mother during its development, and is free of the frond at the proximal end forming a peltate structure (Plates 1a, 18c).

3.3.2.5 The root

The roots of S. polyrrhiza also arise from the node. They are similar to those of all rooted duckweeds in that they are unbranched, non-cutinised, entirely free of root hairs, and possess a conspicuous persistent root cap. S. polyrrhiza produces 5 - 7 roots from each frond. They vary in length from a few to 20 mm and are less than 0·5 mm in diameter. The root cap, approximately 1 mm long, covers the pointed root tip. The root primordia are completely embedded in the nodal tissue, which is pushed out into finger-like sheaths and eventually punctured when the roots elongate (Plate 13b).

In transverse sections of the root, the cells appear to be arranged in concentric rings. Plate 12c shows a transverse section of the mature root near the tip region. The outermost 3 layers of cells comprise the root cap, which consists of large highly vacuolate cells containing plastids. Chloroplasts and amyloplasts were also found in the root of Lemna minor (Wroblewski, 1973). The root cap appears to be attached to the root tip as seen in longitudinal section (Plate 13a). Coming to the root proper, the

outermost layer of cells constitutes the epidermis. The epidermal cells of the root appear to lack chloroplasts at maturity (Plate 12d), so do the parenchyma cells directly beneath, although an ultrastructural investigation would be needed to confirm this. Melaragno (1974) found no chloroplasts in mature epidermal cells in the root of Lemna minor, in contrast to Wroblewski (1973) who found chloroplasts at all developmental stages.

Inside the epidermis are 2 - 3 layers of parenchyma cells comprising the outer cortex (Plate 12c), the outermost layer of cells being much larger than the others at maturity (Plate 12d). The next 2 innermost incomplete layers of cells differentiate into radiating columns 3 cells deep separated by large air spaces. This cylinder of 9 air spaces separates the inner and outer cortex.

The inner cortex consists of a layer of parenchyma cells surrounding the endodermis. Inside the endodermis is the vascular core. The vascular tissue is seen most clearly near the root tip (Plate 12c) and consists of a ring of approximately 10 phloem cells surrounding a single xylem vessel. Two sieve elements and two companion cells occur in a regular arrangement on opposite sides of the central cell. The remaining phloem tissue is composed of phloem parenchyma cells. There are no air spaces betwwen cells which are located within the endodermal layer. The arrangement of vascular tissue described above is extremely similar to that found in Lemna minor (Melaragno and Walsh, 1976), except that the angle made by the sieve elements to the xylem cell in S. polyrrhiza is more acute (~130^{o}) than the arrangement found in L. minor (~160^{o}).

The structure of the immature root primordium is very similar

to that of the root tip, except that the root cap cells are still meristematic in nature (Plate 12a). As the primordium elongates, these cells begin to vacuolate before the rest of the root tissue (Plate 12b).

The functional significance of the roots is difficult to evaluate. The chloroplasts of the root are photosynthetically active (Pirson and Göllner, 1953), but the roots are not essential for nutrient absorption since the fronds grow well under conditions which prevent root elongation (Hillman, 1961), and the entire lower surface of the frond can absorb nutrients from the medium. It has been suggested that they chiefly serve as anchors to keep the fronds upright, and to form the tangled masses found in nature, whose formation protects the individual plantlets from water motion (Arber, 1920).

3.3.2.6 The meristematic pockets

Spirodela polyrrhiza produces daughter fronds from 2 meristematic pockets at the proximal end of the frond. Plate 13b shows a daughter frond emerging from the pocket of a mature mother frond. The daughter is attached to the mother via the stolon which will later abscise from the mother frond. Within the daughter itself, two reproductive pockets are present, each with a daughter frond and its axillary bud to its left, when viewed from the proximal end of the daughter frond. The axillary frond develops when the first daughter has emerged from within the mother pocket. The growing point of the young frond is covered by the prophyllum, a flap of tissue 1 or 2 cells deep. Within each of these daughter fronds, the beginnings of the meristematic pockets can be seen

(Plate 13c). The roots of the frond develop much later than do the young frond primordia. Although the mother frond contains at least 3 generations of daughters, the roots of the mother are only just beginning to elongate (Plate 13b). Below the node five developing roots are present, each with a mature root cap.

The meristematic pocket can also be sectioned longitudinally (Plate 13d). The young frond is completely ensheathed by the prophyllum, and consists almost entirely of meristematic cells which have a large centrally placed nucleus. The nucleus has a well defined nucleolus, which often has a vacuole inside (Plate 14a). The mitochondria are oval in outline (Plate 14b) and the chloroplasts have well developed granal systems (Plate 15a), unusual for meristematic cells. Dictyosomes can also be seen (Plate 15b). The cytoplasm is densely populated with ribosomes (Plate 15d) and microtubules are commonly arrayed along the perimeter of the cell (Plate 15c). Between the cells are numerous plasmodesmata, and mitotic figures are common (Plates 14c, 14d). As development of the young frond continues, the elongation of the stolon pushes the young frond out from the mother pocket (Plate 13d). The typical aerenchyma pattern now forms in the mesophyll with intercellular air spaces being formed between the cells.

Fagerlind and Massalski (1974) report that air space formation in the Lemnaceae results from de-densification of cell wall material, with the formation of small cavities in the wall which are successively replaced by the intercellular space. In plate 16a the developing air space is shown to be filled with granular material. The development of the spongy mesophyll is shown in plates 16a and

16b, where the granular material disappears as the air space approaches maturity.

The upper epidermis of the young frond is still meristematic and actively dividing (Plate 16c) as is the lower epidermis (Plate 16d). Plate 17a shows the lower epidermis of the mother frond, the prophyllum surrounding the young frond and the young frond itself, for purpose of comparison. The cells of the mother are highly vacuolate but the cells of the prophyllum are rather less so and are characterised by their highly irregular outline.

As the cells of the young frond mature the small vacuoles present in the cytoplasm fuse and form a large single one (Plate 17b), and as the cells approach maturity the cytoplasm becomes more diffuse, the nucleolus becomes less prominent and the chloroplasts (Plate 17c) begin to accumulate more starch (Plate 17d).

A detailed analysis of the development of the vegetative frond at the light microscopic level can be found in section 3.5 where it is compared with the development of the turion (section 3•6).

3•4 ORGANISATION OF THE TURION

3.4.1 Gross Morphology

The turions of Spirodela polyrrhiza are smaller and thicker than the vegetative fronds (Jacobs, 1947; Henssen, 1954) being 1•5 - 2•5 mm in length and 2 - 3 mm wide. The turion has the same basic structures including the frond (which is differentiated into a leafy part, a node, and 2 pocket sheaths enclosing the pockets), several primordia and root primordia. Turions are dark olive-green and kidney-shaped (Plate 1c). The upper surface is convex (Plate 3c)

and the lower surface slightly concave. It was found that the pigmentation and size of the turion depended much on the condition of the producing mother frond; a large non-senescing mother producing a large, dark turion (cf. Jacobs, 1947).

At the proximal end is a rather obvious pocket sheath which is lighter in colour (Plate 3d). The structure of the pocket sheath is best seen with the scanning electron microscope (Plate 6a). The sheath is much thicker than in the vegetative frond, but is also larger on one side. Turions are produced from the mother pockets in the same way as vegetative fronds (Plate 18c) but the stolon does not elongate. When the turion is mature, it abscises from the mother and sinks; in contrast to the vegetative fronds which remain associated in plantlets. The dormant turion has no functional roots, but possesses up to 6 root primordia which remain embedded in the tissue below the node (Plate 18a).

3.4.2 Anatomy

A transverse section of the turion (Plate 18a) reveals once again the 3 basic tissue types.

The epidermal layers of the mature turion appear similar to those of the vegetative frond, but the cells are heavily pigmented with anthocyanin, especially the upper epidermis. Under the electron microscope however, they appear very different. They are much less vacuolated than those found in the vegetative frond and have an extremely granular cytoplasm and large starch grains (Plate 19a). The cytoplasm has a great deal of rough endoplasmic reticulum arrayed in large stacks (Plate 19b). The chloroplast is distorted due to the large amount of accumulated starch (Plate 19c). Stomata

are also present in the upper epidermis of the turion (Plate 19d), but they also have numerous starch grains and appear to be closed (Plate 7d). This is shown more strikingly under the scanning electron microscope, where a turion still attached to its mother frond is shown in plate 6b. The stomata of the vegetative frond are widely open whereas those of the turion are shut (Plates 6c and 6d).

The lower epidermis of the turion also appears quite normal under the light and scanning electron microscopes (Plate 18d), but the cells are also full of starch grains and have a fairly dense cytoplasm (Plate 20a).

The mesophyll is entirely undifferentiated with very few intercellular air spaces. The cells are so encumbered with starch that few other organelles can be distinguished at the light microscopic level (Plate 18b). The mesophyll has an unusually high number of raphide cells and idioblasts, and most of the cells have large globules of darkly staining material in their vacuoles (Plate 21a). The mesophyll cells, like the epidermal cells, appear to be less vacuolate than the mature mesophyll cells of the vegetative frond (Plate 20b). This finding was confirmed by stereological analysis (section 3•7).

Although the chloroplasts are distorted by the presence of the large starch grains, in most cases the chloroplast envelope appears to be intact (Plate 20c). The nucleus also has an intact envelope and a well defined nucleolus (unlike the mature mesophyll cell of the vegetative frond). Mitochondria are numerous and myelin figures, which were also seen in meristematic vegetative frond cells, are common (Plate 20d). Because of the extremely granular nature

of the cytoplasm (probably ribosomes), the endoplasmic reticulum and dictyosomes are difficult to discern.

Interestingly the vascular tissue of the turion is identical to that found in the vegetative frond (Plate 21b) and the cells show none of the striking characteristics of the rest of the mesophyll; with the chloroplasts containing very little starch (Plates 22a and 22b).

The mother fronds associated with the turions are also less sensitive to the effects of ABA. Starch does accumulate in the chloroplasts of the mother fronds of S. polyrrhiza (UC) (Plate 23c), but to a much lesser extent than in the turion. It was interesting also to find that in S. polyrrhiza (N), which formed larger and more vigorous turions, the mother frond chloroplasts contained hardly any starch at all (Plate 23d).

At the proximal end of the turion (Plate 18a) can be seen the very prominent pocket sheath and 3 root primordia embedded in the nodal tissue. The turion also has 2 reproductive pockets, and the largest pocket contains the first daughter. This is about 0·4 mm or less in length and sufficiently well developed to contain primordia of the next generation in its pockets were it a vegetative frond primordium. But the turion primordium is dormant, and the pockets are still undifferentiated. A small axillary bud is usually present in this pocket, but this rarely develops, as the turion usually dies after the first frond has emerged in germination. The other pocket of the turion contains the second primordium and rarely the fourth.

The first turion primordium consists entirely of meristematic

cells (Plate 22c), which appear very similar to the cells of a primordium of a vegetative frond. There are no starch grains in the primordium. However, the cells of the prophyllum, although not highly vacuolate, have many starch grains (Plate 22d). For comparison the turion, the prophyllum, and the turion primordium are shown together in plate 23a.

The structure of the turion appears to be well organised with respect to its function. Its smaller size and undifferentiated mesophyll with no air spaces allow the turion to sink rapidly once free of the mother frond, thus allowing it to overwinter in the deeper warmer layers of the water. The numerous starch grains would also add to the overall specific gravity, but their main function is probably to provide a store of energy for the growth of the first primordium when the turion germinates.

3.4.3 The Semi-turion

The semi-turion, its formation discussed in section 3.2.2, is not only morphologically turion at its proximal end and vegetative frond at its distal end, but its internal anatomy also reflects this sharp division (Plate 21c). The pigmented proximal half of the semi-turion has closely packed cells with very few air spaces in between (Plate 21d); whereas within a few microns the green distal half shows aerenchyma development (Plate 21e). The cells of the distal half of the semi-turion have not however escaped all of the effects of ABA, since they are also heavily loaded with starch grains. Since the cells of the distal half are older than those of the proximal pigmented half, it seems likely that the developing cell

has a wider sensitivity to ABA with respect to starch formation than to anthocyanin accumulation.

3.5 DEVELOPMENT OF THE VEGETATIVE FROND

The developmental stages in the production of the vegetative frond can be seen in plates 24 - 30.

The young bud (Plate 24a) consists entirely of an undifferentiated cluster of meristematic cells, increasing in size by cell division alone. As the bud begins its development (Plate 24b) it is connected with the mother frond by a very short and still meristematic stalk or stolon. The growing point of this bud is covered by the prophyllum, which is joined to the stolon at the base of the bud, thus sheathing the bud completely. By the time the bud is approximately 0·2 mm and 26 cells long (cf. Rimon and Galun, 1968) (Plate 24c), the cells have increased in size and small vacuoles have formed, predominantly in the lower cell layers of the bud.

The bud continues to expand by both cell division and expansion, the prophyllum is ruptured and vascular tissue begins to differentiate at various points along the length of the young frond (Plate 25a). Most of the cells of the young frond appear essentially meristematic until the frond is approximately 0·45 mm long (Plate 25b). Here the cells of the lower distal portion of the frond have formed fairly large vacuoles and a few intercellular air spaces have developed. The palisade and spongy mesophyll layers now become distinct at the distal end, although neither stomata nor vascular tissue have yet matured. Even when the frond reaches 0·55

mm in length (Plate 26a) the majority of the cells are meristematic in nature, although the terminal spongy mesophyll cells are nearly mature and air space formation is also apparent. It therefore appears that not only do the distal cells of the frond mature first (cf. Maksymowych, 1973 on Xanthium leaves), but the spongy mesophyll appears to differentiate well before the palisade layers. By the time the frond is nearly 0·65 mm long, the mesophyll has differentiated throughout the length of the frond and immature stomata are present in the upper epidermis. The upper cell layers still appear to be actively dividing (Plate 26b). As development continues, the elongation of the stolon pushes the young frond out from under cover of the meristematic pocket of the mother frond.

The prophyllum is stretched and fused along the lower side of the axis, forming the lower flap of the left hand pocket. The growing point, which in early stages was surrounded by the prophyllum, expands to form the broad leafy part of the plant. The backward growth of the proximal dorsal margin of the frond forms the upper cover of both pockets, while a meristematic centre is retained at the node. In the axil of the prophyllum which, with the proximal part of the frond, forms the left hand pouch, is an axillary bud, which will develop into a new frond when the older frond has abscised from the mother pocket. The stolon connecting the developing frond can be seen more clearly in plate 27. The cells of the stolon are meristematic and differ from the meristematic cells of the young frond in having spindle-shaped chloroplasts. This frond is nearly 0·7 mm long and its distal end just reaches the margin of the mother pocket. Stomata are easily recognisable in the

upper epidermis, but the meristematic pockets of the young frond are not visible since they are not in the same plane as the stolon. One of the meristematic pockets can be seen in the slightly longer developing frond in plate 28. Embedded at the node is a root primordium. The roots generally do not elongate until the frond is about 4 mm long. The vascular tissue is now well defined, and the xylem elements are easily recognisable in oblique section.

The majority of the frond has developed aerenchyma tissue in the 0·85 mm frond shown in plate 29, although some of the palisade cells in the proximal half of the frond remain relatively unvacuolated. The axillary bud above the point of attachment of the stolon is clearly seen in this section, and this bud has its own axillary bud in its axil, so that 3 successive daughters are contained within the mother pocket.

The longest vegetative frond that could be successfully sectioned intact is shown in plate 30. This frond is nearly 1·3 mm long and the tissue is fairly well differentiated. The sieve elements in the vascular bundles have already begun to accumulate cell wall material. Cell division is probably completed by this stage, and the frond will eventually enlarge to up to 7 mm long mainly by cell expansion and by intercellular air space formation.

3.6 DEVELOPMENT OF THE TURION

The developmental stages in the production of the turion can be seen in plates 31 - 35. The turion primordium is morphologically indistinguishable from that of the vegetative frond until it is at least 0·45 mm long (Plate 32b). The length at which the 2 types of

primordia become recognisable could not be determined with absolute confidence, since even similarly sized sections of vegetative fronds often appeared to differ anatomically to some degree. My observations were based mainly on the fact that in all sections of fronds 0•45 mm or longer, the layer of cells just above the lower epidermis had fairly large vacuoles, especially near the distal end of the frond, indicating the onset of the main period of cell expansion. However, only in vegetative fronds was this process also accompanied by extensive intercellular air space formation and cell separation.

Above 0•55 mm the developing turion is easily recognisable. The cells of the developing turion often have darkly staining inclusions, although starch grains are not yet apparent with the light microscope (Plate 33). The thickness of the frond is also diagnostic of the destiny of the frond at this stage, with the developing turions being some 30 - 50% thicker than their vegetative counterparts of the same length. By this time the turion also possesses the very large lower pocket sheath so characteristic of the mature turion.

The developing turion continues to expand, although at a slower rate than the developing frond (since the fully mature turion rarely exceeds 2 mm in length) and the cells in the distal half accumulate starch grains visible in the light microscope when the frond is approximately 0•66 mm long (Plate 34). A nearly half grown turion is shown in plate 35. Most of the cells of the developing turion have accumulated large starch grains and a few of the cells, already devoid of cytoplasmic contents, are filled with tannin-like material. There is no differentiation of the mesophyll and the root primordia

lie embedded in the nodal tissue. It should be noted that the developing turion shown in plate 35 is of approximately the same length as the vegetative frond in plate 30.

3.7 STEREOLOGICAL COMPARISON OF FROND AND TURION TISSUE

Although the cells of the mature turion are approximately the same size as those of the vegetative frond, they have a very different fine structure. In some respects the turion cells appear not to have reached the same level of maturity; characterised by less vacuolation, a denser cytoplasm and a nucleus with a prominent nucleolus. At the expense of the normal maturity programme, the turion cells have accumulated starch and secondary products like anthocyanin.

However, these observations have so far been deduced from rather subjective analysis of the tissue. In ultrastructural examination of a tissue, it is all too easy to concentrate one's observations to the more aesthetic parts of the cell, and only record particularly interesting features e.g. organelle structure as opposed to the ground cytoplasm or the vacuole. Whilst this approach is perfectly sound in purely descriptive work, the information obtained is of limited use in measuring and quantifying ultrastructural differences between tissues or treatments.

In this study for example, one can say with some confidence that the turion contains more starch than the vegetative frond, because this is such a striking feature. However, is there really more ground cytoplasm in the cells of the turion or does the large amount of accumulated starch make this only appear to be true? This

question, and many others, can only be answered confidently by a full stereological analysis of the 2 types of tissue, turion and vegetative frond.

Stereological analysis was performed on vegetative fronds and mature turions in order to quantify the structural differences between the tissues. It was hoped that an understanding of these differences would help in the interpretation of the developmental changes occurring during turion formation.

3.7.1 Definitions

To define the morphometric model, the tissue is systematically sub-divided into compartments, each of which forms a structural entity. These compartments are schematically represented in figure 15 with an indication of their stereological nomenclature. For the purposes of this study a number of simplifications and conventions are introduced:

a) the cell is considered to consist of the cell wall, the vacuole and the protoplasm,

b) the protoplasm contains the cytoplasm, the nucleus and starch grains,

c) the nuclear envelope is considered as part of the nucleus,

d) to avoid complication the term 'endoplasmic reticulum' is here understood to include all membrane-bounded cytoplasmic cisternae, tubules, and vesicles including the dictyosomes, but excluding the nuclear membrane,

e) starch grains are not considered to be an integral part of the chloroplast, and hence cytoplasm, but are included in the protoplasm for convenience only, and

Figure 15

Schematic representation of the plant cell defining the various compartments on the left and the measured stereological parameters on the right.

V = volume density, S = surface density

f = frond

a = air space

c = cell

cw = cell wall

v = vacuole

p = protoplasm

st = starch

n = nucleus

cy = cytoplasm

gc = ground cytoplasm

er = endoplasmic reticulum

mi = mitochondrion

ch = chloroplast

ce = chloroplast envelope

ct = chloroplast thylakoid

cs = chloroplast stroma

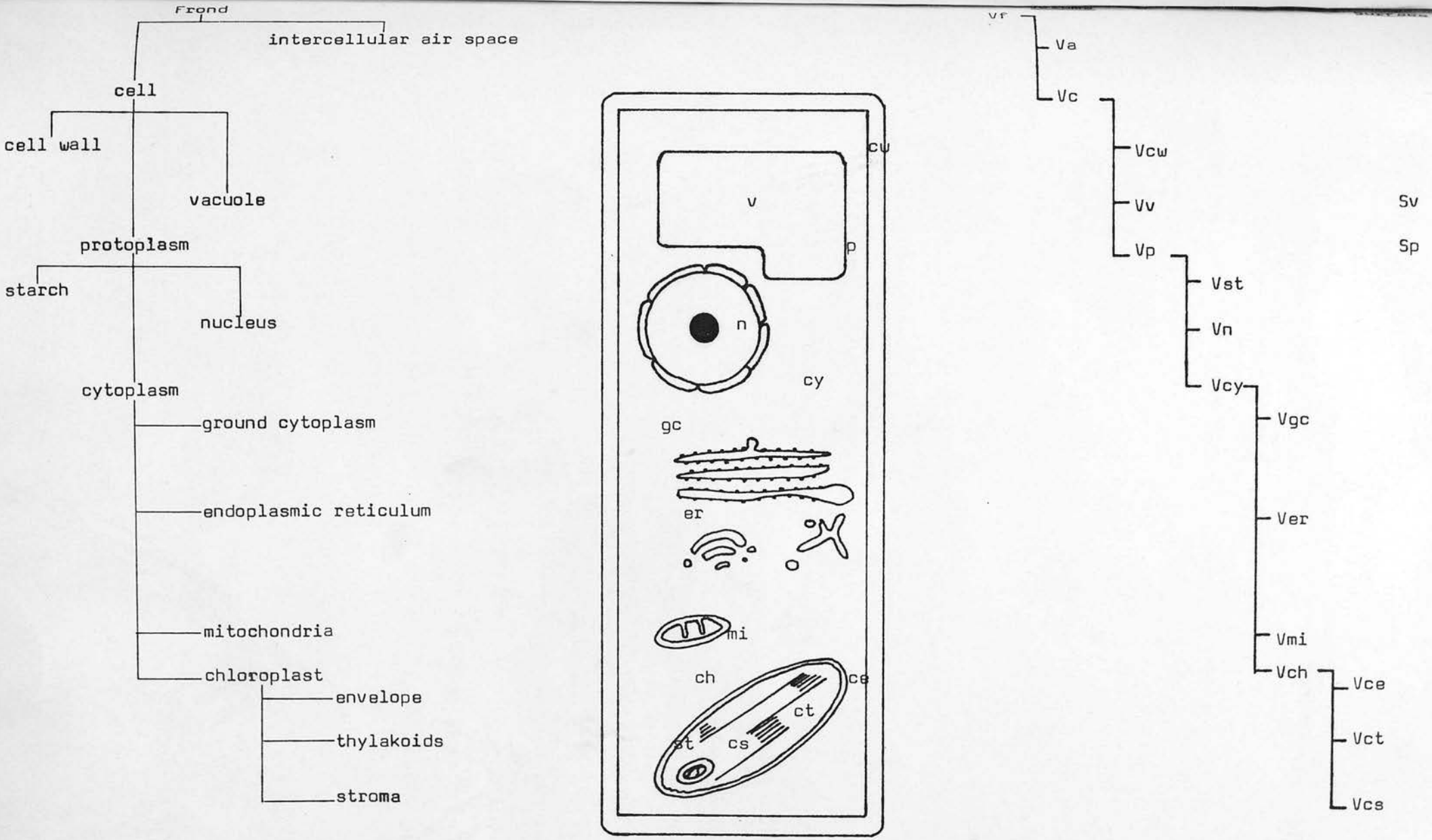

Frond
intercellular air space
cell
cell wall
vacuole
protoplasm
starch
nucleus
cytoplasm
ground cytoplasm
endoplasmic reticulum
mitochondria
chloroplast
envelope
thylakoids
stroma
cu
v
p
n
cy
gc
er
mi
ch
ce
ct
cs
st
Vt
Va
Vc
Vcw
Vv
Vp
Vst
Vn
Vcy
Vgc
Ver
Vmi
Vch
Vce
Vct
Vcs
Sv
Sp

FIGURE 15

f) the term 'ground cytoplasm' is defined as the cytoplasmic space extending from the plasmalemma to the surface of membrane-bounded organelles. It thus includes free and bound ribosomes.

The tissue was examined at 3 levels of magnification.

3.7.2 Level I (Magnification 1,090X)

This was a low power study to determine the volume of frond and turion tissue occupied by air spaces (Va), cells (Vc), protoplasm (Vp), vacuoles (Vv) and cell walls (Vcw). 48 point counts were made on each of 25 photomicrographs for both tissue types (Plate 36). Table 2 shows the volume densities obtained for each compartment. It can be seen that frond tissue has over twice the volume of air space (29%) as the turion tissue (12%). Undoubtably if a comparison had been made between only fully mature fronds and turions, this difference would be even greater. However, the comparison was made between whole plantlets and turions, since this comparison correlates better with the biochemical studies.

Correspondingly the volume density of cells in the turion is higher (88%) than in frond tissue (72%). Within the cell the largest compartment in frond tissue is the vacuole which takes up 66% of the total cell volume. The turion cell has a vacuole half the size, comprising only 35% of the cell volume. This difference is compensated not only by an increase in the protoplasmic volume from 30 - 56% (in frond and turion respectively), but also an increase in the volume of cell wall material from 4 to 10%.

This level of magnification proved to be the most accurate for the measurement of cell and air space volume densities, adequate for

Table 2: Stereological analysis at Level I

a)

Compartment	Frond (cm^3/ cm^3 tissue)	Turion (cm^3/ cm^3 tissue)	
air space (Va)	0•2850 ± 0•0287	0•1209 ± 0•0079	***
cell (Vc)	0•7150 ± 0•0287	0•8792 ± 0•0079	***

b)

Compartment	Frond (cm^3/ cm^3 cell)	Turion (cm^3/ cm^3 cell)	
protoplasm (Vp)	0•2995 ± 0•0300	0•5581 ± 0•0187	***
vacuole (Vv)	0•6644 ± 0•0272	0•3474 ± 0•0206	***
cell wall (Vcw)	0•0361 ± 0•0073	0•0965 ± 0•0111	***

a) The differences between the volume densities of tissue compartments in frond and turion tissue of S. polyrrhiza (N).

b) The differences between the volume densities of cellular compartments in frond and turion tissue of S. polyrrhiza (N).

Values are the mean of 25 determinations and are shown ± the standard error of the mean. Level of significance of differences by the Rank Sum Wilcoxon test * 10% ** 5% *** 1%.

the major cellular compartments, but too low a magnification for the further resolution of the protoplasm.

3.7.3 Level II (Magnification 2,703X)

This level was used mainly to determine the volume densities of the major cell compartments; the protoplasm (Vp), the vacuole (Vv) and the cell wall (Vcw). Although these measurements were made at level I, the resolution obtained with the electron microscope is far superior. 42 point counts (level IIa) were made on each of 20 electron micrographs for both tissues (Plate 37a, 37c). At this level it was also possible to distinguish between the nucleus (Vn), the cytoplasm (Vcy) and the starch grains (Vst). This level was also used to calculate the surface density of the plasmalemma (Sp) and the tonoplast (Sv), since the magnification is low enough to avoid bias, but high enough to resolve the limits of the proroplast and the vacuole. 210 point and 105 intersection counts (level IIb) were made on the same electron micrographs (Plate 37b, 37d).

Table 3 shows the volume and surface densities obtained for each compartment. At level IIa (Table 3a) the values for the volumes of the protoplasm and the vacuole compare very favourably to those obtained at level I in the frond tissue. In the turion however, the vacuolar volume is higher (and the protoplasmic volume lower) than the values obtained at level I; since the many small vacuoles in the turion were not detected at the lower magnification. The overall pattern is nevertheless very similar, with the vacuolar volume altering from 68% in frond tissue to 42% in the turion. The cell wall was also better resolved at this level, still showing

Table 3

a) The differences between the volume densities of the major cellular compartments in frond and turion tissue at Level IIa in Spirodela polyrrhiza (N).

b) The differences between the volume densities of the cellular compartments and the surface densities of the membranes of frond and turion tissue in S. polyrrhiza (N) at Level IIb.

All values are the means of 20 determinations ± the standard error of the mean. Level of significance of differences by the Rank Sum Wilcoxon test * 10% ** 5% *** 1%.

Table 3: Stereological analysis at Level II

a)

Compartment	Frond	Turion	
	(cm^3/ cm^3 cell)		
protoplasm (Vp)	0•2825 ± 0•0217	0•4586 ± 0•0279	***
vacuole (Vv)	0•6735 ± 0•0251	0•4347 ± 0•0267	***
cell wall (Vcw)	0•0456 ± 0•0093	0•1053 ± 0•0106	***

b)

Compartment	Frond	Turion	
	(cm^3/ cm^3 cell)		
protoplasm (Vp)	0•2672 ± 0•0236	0•4625 ± 0•0167	***
vacuole (Vv)	0•6834 ± 0•0256	0•4238 ± 0•0197	***
cell wall (Vcw)	0•0502 ± 0•0038	0•1126 ± 0•0072	***
cytoplasm (Vcy)	0•2572 ± 0•0227	0•2270 ± 0•0152	
starch grains (Vst)	0•0000 ± 0•0000	0•2308 ± 0•0091	***
nucleus (Vn)	0•0100 ± 0•0019	0•0057 ± 0•0013	
	(cm^2/ cm^3 cell)		
plasmalemma (Sp)	3554 ± 303	3263 ± 184	
tonoplast (Sv)	3140 ± 162	3100 ± 127	

slightly over twice the amount of cell wall material surrounding the cells of the turion. This increase in cell wall volume can at once be seen in plates 37c and 37d, where the cells of the turion have much thicker cell walls than those of frond cells.

Table 3b shows that approximately half of the volume of the protoplasm in the turion cell is occupied by starch grains. Since there was no starch in the frond tissue studied, the volume density of starch present in the turion represents an increase of 23%. There was no difference in the cytoplasmic or nuclear volume between control and turion tissue ($Vcy \approx 24\%$; $Vn \approx 1\%$).

Measurements made at level IIa were the most accurate for the determination of the major cellular compartments i.e. Vp, Vv and Vcw; and those made at level IIb for the further resolution of the protoplasm into Vcy, Vst and Vn, since the test points are nearer together at this level which results in a more accurate estimation of smaller or rarer components (Weibel, 1973). The surface densities of the plasmalemma and tonoplast were also calculated at this level, but no difference in the surface area of either of these membranes between the tissues was found.

3.7.4 Level III (Magnification 27,030X)

The volume densities of ground cytoplasm (Vgc), endoplasmic reticulum (Ver), mitochondria (Vmi) and chloroplasts (Vch) were estimated. The volume composition of the chloroplast was also determined in terms of its component parts; chloroplast envelope (Vce), thylakoid lamellae (Vct) and stroma (Vcs). 210 point counts were made on each of 20 electron micrographs (Plate 38) and the

results are shown in Table 4.

At level IIb it was established that the cells of the turion do not contain a higher proportion of cytoplasm, and this is reflected by the analysis of the cytoplasm into its component parts at level III. In frond tissue approximately 37% of the cytoplasm is composed of ground cytoplasm and 56% of chloroplasts. The endoplasmic reticulum and mitochondria comprise 2% and 5% respectively of the total cytoplasmic volume. The cytoplasm of the turion is similarly subdivided with 42% ground cytoplasm, 49% chloroplast, 1% endoplasmic reticulum and 7% mitochondria. The volume distribution of the component parts of the chloroplast is also very similar in both samples, the chloroplast being 68% stroma, 25% thylakoid lamellae, 4% envelope, 2% spherical body (see below) and 0·2% plastoglobuli in the frond sample. Although no plastoglobuli were detected within the sampling area, plastoglobuli were also seen in the chloroplasts of turion cells.

However, during the course of this study, the distribution of one particular 'organelle' proved very interesting. In the chloroplasts of frond samples, numerous spherical bodies were observed (Plate 23b). These are much larger than plastoglobuli and are less electron dense. Their surface appears to be cratered rather like the surface of the moon, and they possess no membrane. They do not resemble the membrane-bounded bodies of chloroplasts described in the literature (Henry, 1975), but appear more similar to the lipid droplets found in the cytoplasm of plant cells (Gunning and Steer, 1975). While these bodies are usually found within the stroma of the chloroplast in frond tissue, they are only found in

Table 4: Stereological analysis at Level III

Compartment	Frond (cm^3/ cm^3 cytoplasm)	Turion (cm^3/ cm^3 cytoplasm)
ground cytoplasm (Vgc)	0•3727 ± 0•0297	0•4209 ± 0•0437
endoplasmic reticulum (Ver)	0•0187 ± 0•0057	0•0088 ± 0•0030
mitochondria (Vmi)	0•0536 ± 0•0117	0•0723 ± 0•0178
chloroplast (Vch)	0•5556 ± 0•0337	0•4858 ± 0•0532
chloroplast envelope (Vce)	0•0222 ± 0•0036	0•0171 ± 0•0067
chloroplast stroma (Vcs)	0•3791 ± 0•0231	0•3279 ± 0•0399
thylakoid lamellae (Vct)	0•1405 ± 0•0126	0•1409 ± 0•0166
spherical bodies (Vsb)	0•0125 ± 0•0044 in chloroplast	0•0122 ± 0•0050 in ground cytoplasm
osmiophilic granules (Vos)	0•0013 ± 0•0009	0•0000 ± 0•0000

The differences between the volume densities of the cytoplasmic compartments in frond and turion tissue in S. polyrrhiza (N). All values are the means of 20 determinations ± the standard error of the mean. Level of significance of differences by the Rank Sum Wilcoxon test * 10% ** 5% *** 1%.

the ground cytoplasm of turion tissue (Plates 20 and 21). These bodies were never found in the cytoplasm of untreated vegetative fronds or in the chloroplasts of turions.

The major differences in the volume composition of frond and turion tissue lie mainly in the major cell compartments (Fig. 16). Although the two cell types have similar amounts of cytoplasm, the turion cells have a much larger proportion of extra-cytoplasmic material i.e. starch and cell wall. If starch is accumulated as a source of energy for the germination process, then the thicker cell wall may also act as a store for other polysaccharides, proteins and perhaps calcium. This increase in extra-cytoplasmic material in the turion appears to be mainly at the expense of the volume of the vacuole.

Bearing in mind the similar sizes of the two cell types and the similar volume composition of the cytoplasm, the tissues differ ultrastructurally only in 3 main respects; air space formation, vacuolation, and starch and cell wall material accumulation. This indicated that the development of the turion cell and the vegetative frond cell are initially quite similar i.e. cell division phase. Only in the cell elongation phase is the diverging point for both developmental programmes likely to occur. The vegetative frond cell matures in the normal fashion, producing a large central vacuole surrounded by a thin layer of cytoplasm. The cells separate from one another with the formation of intercellular air spaces. The turion cell however, accumulates large amounts of starch and cell wall material during the period of vacuolation, thus expanding the cytoplasm in all directions at the expense of vacuolar expansion.

Figure 16

Schematic representation of the data obtained by stereological analysis. The histograms show the volume fraction of the various tissue, cell, cytoplasm and chloroplast compartments in both frond and turion tissue of S. polyrrhiza (N).

a) Volume composition of tissue

- cell
- air space

b) Volume composition of cells

- vacuole
- cytoplasm
- starch
- cell wall
- nucleus

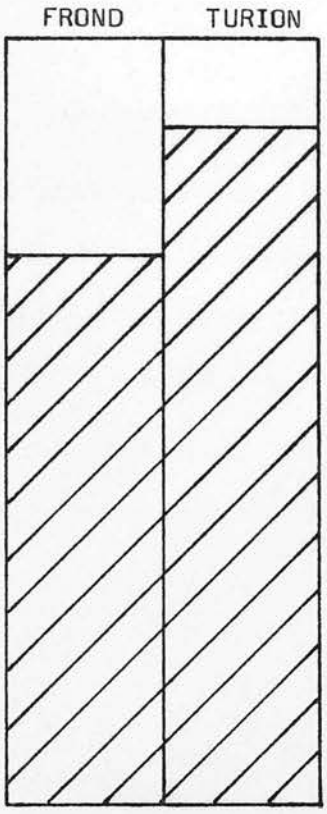
c) Volume composition of cytoplasm

- ground cytoplasm
- chloroplast
- mitochondrion
- endoplasmic reticulum
- spherical body

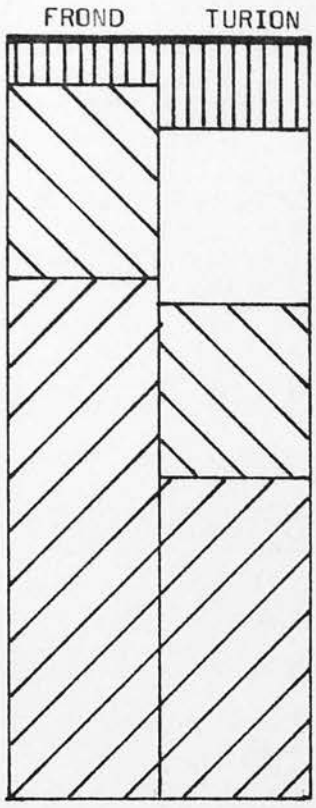
d) Volume composition of chloroplast

- stroma
- thylakoid
- envelope
- spherical body

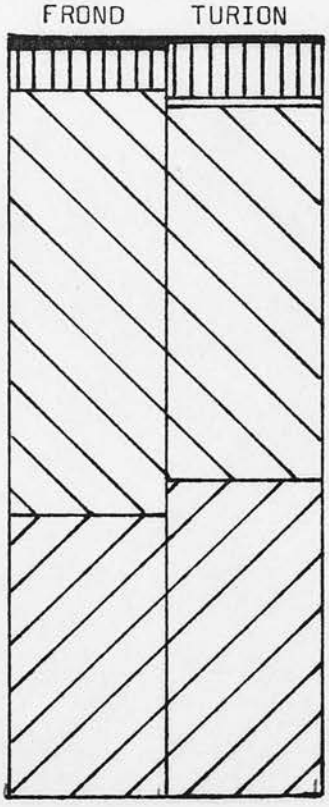
FIGURE 16



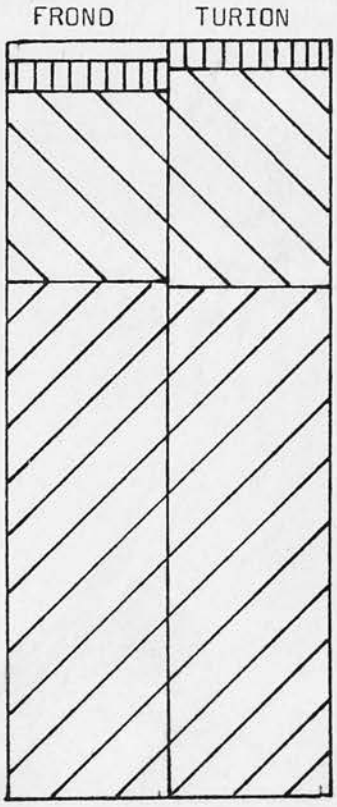
(a)



(b)



(c)



(d)

Czopek (1967) has shown that the dormant turion has only 25% of the photosynthetic capacity of the vegetative frond, and although the chloroplasts of frond and turion cells were similar in terms of their volumetric composition, the large amount of starch contained within the turion chloroplast would, I suspect, severely interfere with their photosynthetic capacity.

All the stereological data obtained was calculated in units of $cm^3 . g^{-1}$ of tissue in table 5.

Table 5: Summary of stereological analysis

Compartment	Frond			Turion		
	$cm^3 \cdot g^{-1}$	$cm^3 \cdot cm^{-3}$ cell	$cm^3 \cdot cm^{-3}$ cytoplasm	$cm^3 \cdot g^{-1}$	$cm^3 \cdot cm^{-3}$ cell	$cm^3 \cdot cm^{-3}$ cytoplasm
Vf	1·0000	-	-	1·0000	-	-
Va (I)	0·2850	-	-	0·1209	-	-
Vc (I)	0·7150	-	-	0·8792	-	-
Vcw (IIb)	0·0359	0·0502	-	0·0926	0·1053	-
Vv (IIb)	0·4886	0·6834	-	0·3726	0·4238	-
Vp (IIb)	0·1910	0·2672	-	0·4066	0·4625	-
Vst (IIb)	0·0000	0·0000	-	0·2029	0·2308	-
Vn (IIb)	0·0072	0·0100	-	0·0050	0·0057	-
Vcy (IIb)	0·1839	0·2572	-	0·1996	0·2270	-
Vgc (III)	0·0687	0·0959	0·3727	0·0840	0·0955	0.4209
Ver (III)	0·0034	0·0048	0·0187	0·0018	0·0020	0.0088
Vmi (III)	0·0099	0·0138	0·0536	0·0144	0·0164	0.0723
Vch (III)	0·1022	0·1429	0·5556	0·0974	0·1108	0·4858
Vce (III)	0·0041	0·0057	0·0222	0·0034	0·0039	0·0171
Vct (III)	0·0258	0·0361	0·1405	0·0281	0·0320	0·1409
Vcs (III)	0·0697	0·0975	0·3791	0·0654	0·0744	0·3279
Vsb (III)	0·0023	0·0032	0·0125	0·0024	0·0028	0·0122
Vos (III)	0·0002	0·0003	0·0013	0·0000	0·0000	0·0000

All the volumetric data have been converted to units of $cm^3 \cdot g$ fresh weight of tissue^{-1}. V = volume density, f = frond, a = air space, c = cell, cw = cell wall, v = vacuole, p = protoplasm, st = starch, n = nucleus, cy = cytoplasm, gc = ground cytoplasm, er = endoplasmic reticulum, mi = mitochondrion, ch = chloroplast, ce = envelope, ct = thylakoid, cs = stroma, sb = spherical body, os = plastoglobulus.

CHAPTER 4

RESULTS AND DISCUSSION

THE BIOCHEMICAL BASIS OF TURION FORMATION

4.1 FRESH AND DRY WEIGHT LEVELS DURING TURION FORMATION

Dry weight measurement was found to be unsatisfactory for the estimation of growth inhibition of Spirodela polyrrhiza by ABA, since far less inhibition was detected as compared with fresh weight or frond number estimation (section 3.1). When fresh and dry weights of the cultures were expressed on a frond basis, it was apparent that although frond production was severely inhibited by ABA, dry weight per frond actually increased. Noticeably this increase was related to the ABA concentration (Figs. 17 and 18).

The % increase in dry weight per frond in S. polyrrhiza is even higher than that reported for the non-turion forming species Lemna minor (McLaren and Smith, 1976), where 10^{-6} M ABA caused an ~2 fold stimulation in dry weight per frond; this compares with a 3 fold increase for S. polyrrhiza (UC) and a 4 fold increase for S. polyrrhiza (N) at the same concentration. Fresh weight per frond decreased noticeably around turion inducing concentrations of ABA, probably because ABA causes the production of smaller fronds and/or turions. This inhibition of the fresh weight per frond is less striking at very high concentrations of ABA, presumably since no new fronds are formed. Any small decrease in the fresh weight of these fronds not attributable to ABA might possibly result from the onset of senescence.

It has been reported in the literature that ABA increases the starch content of guard cells (Mansfield and Jones, 1971), of roots of L. minor (Newton, 1974, 1977), and in whole plantlets of L. minor (McLaren and Smith, 1976). My own ultrastructural observations have

Figure 17

The effect of ABA concentration on the fresh and dry weight levels per frond of S. polyrrhiza (UC) after 8 days incubation.

○ Fresh weight per frond as % control

● Dry weight per frond as % control

□ Dry/fresh weight ratio (%)

FIGURE 17

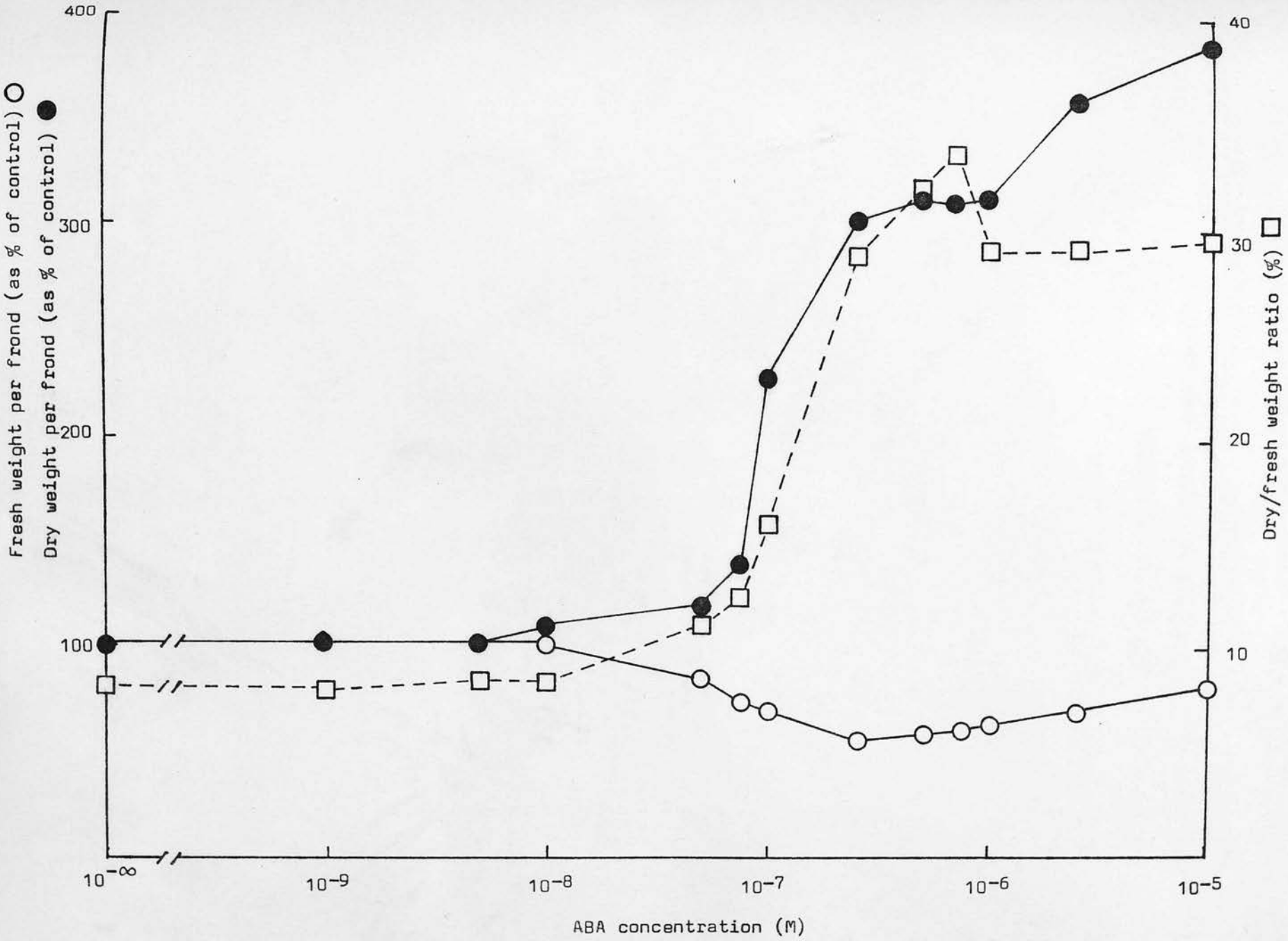

Fresh weight per frond (as % of control) ○
Dry weight per frond (as % of control) ●
Dry/fresh weight ratio (%) □
400
300
200
100
40
30
20
10
$10^{-\infty}$
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
ABA concentration (M)

Figure 18

The effect of ABA concentration on the fresh and dry weight levels per frond of *S. polyrrhiza* (N) after 8 days incubation.

○ Fresh weight per frond as % control

● Dry weight per frond as % control

□ Dry/fresh weight ratio (%)

FIGURE 18

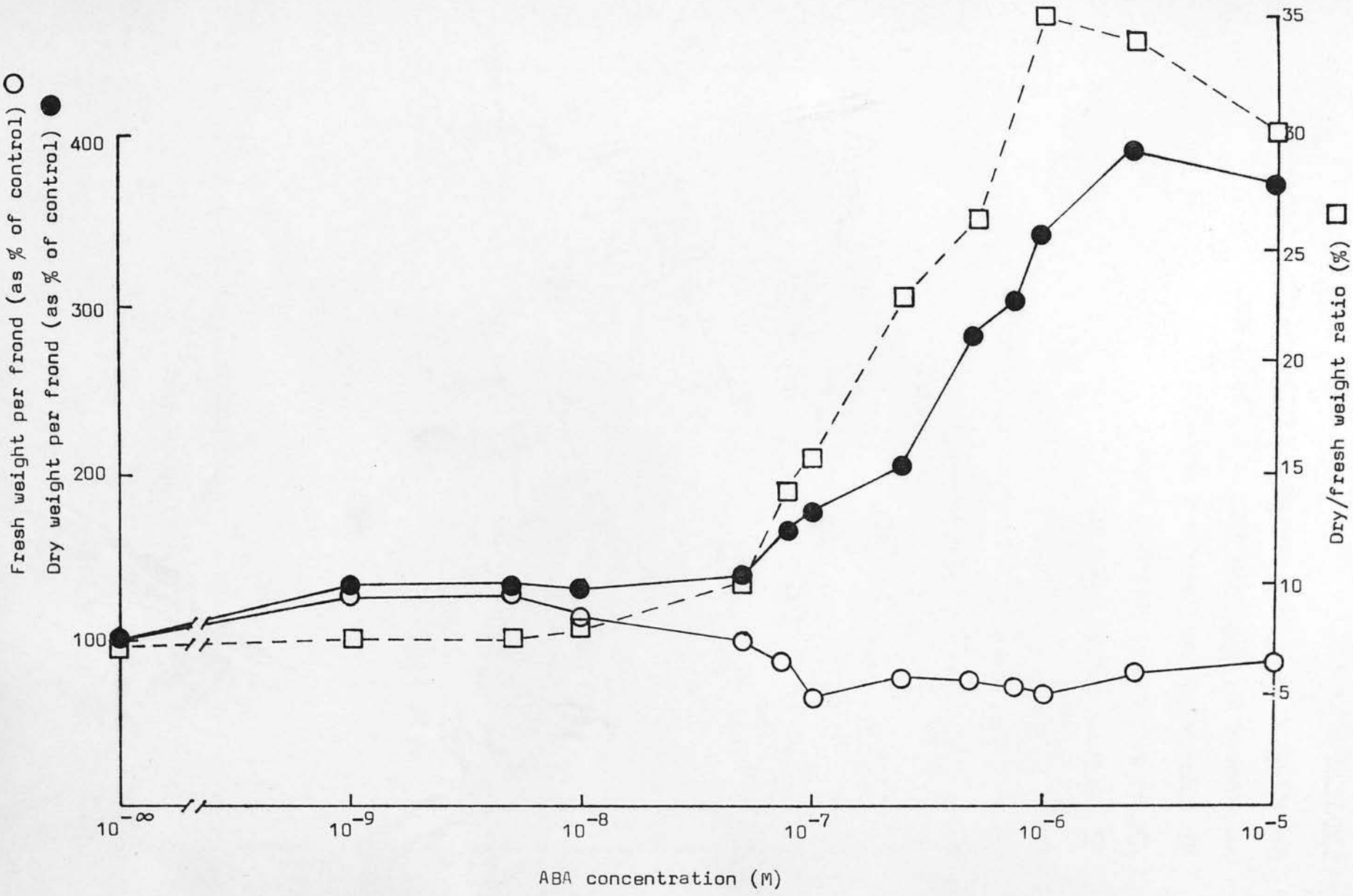

Fresh weight per frond (as % of control) ○
Dry weight per frond (as % of control) ●
Dry/fresh weight ratio (%) □
400
300
200
100
35
30
25
20
15
10
5
$10^{-\infty}$
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
ABA concentration (M)

Figure 19

The effect of incubation time on the fresh and dry weight levels per frond of S. polyrrhiza (UC) in 5×10^{-7} M ABA.

○ Fresh weight per frond as % control

● Dry weight per frond as % control

□ Dry/fresh weight ratio (%)

Actual levels:

Fresh weight. frond^{-1} = 1714 → 1491 µg. frond^{-1}

Dry weight. frond^{-1} = 119 → 321 µg. frond^{-1}

FIGURE 19

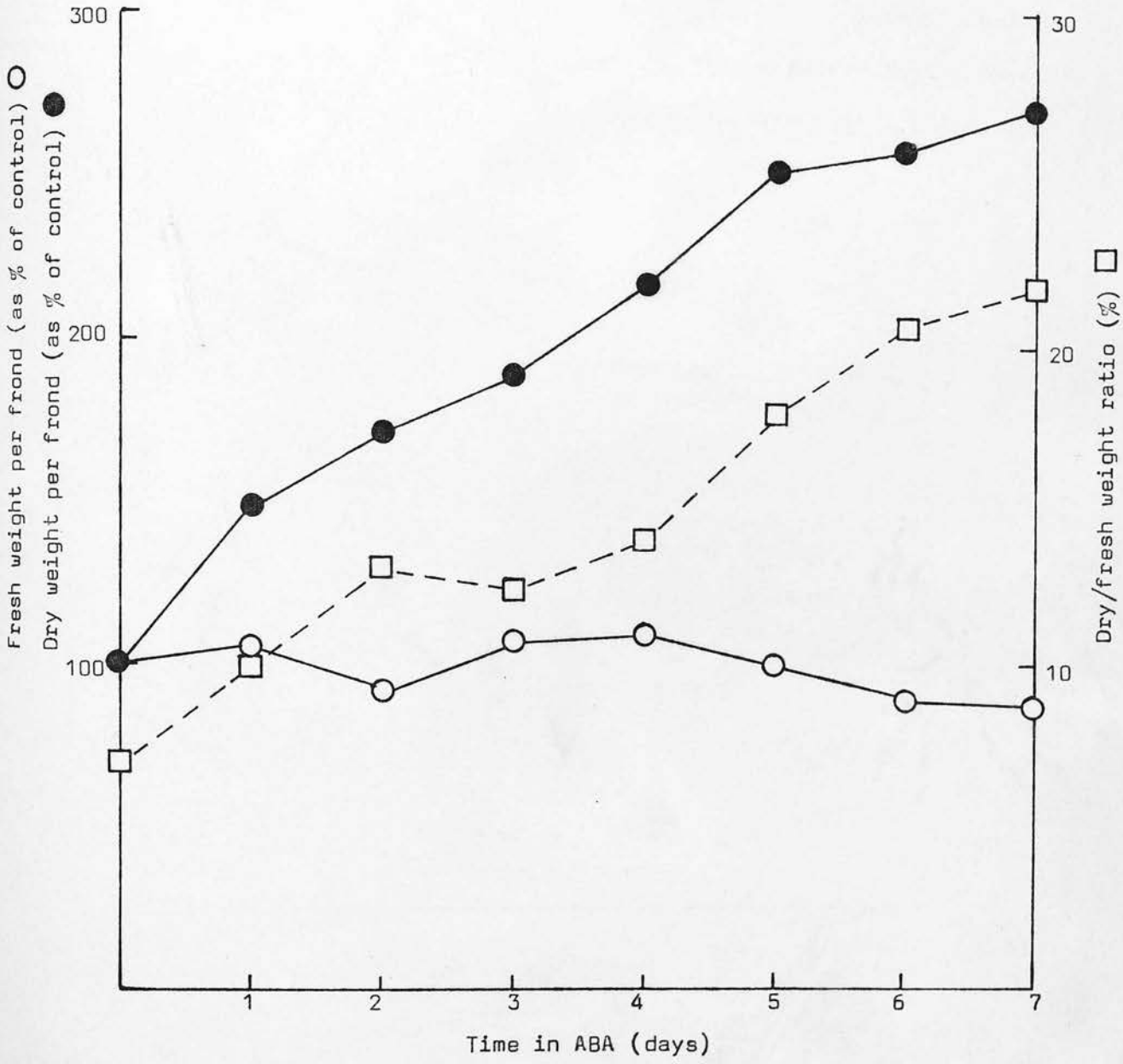

Fresh weight per frond (as % of control) ○
Dry weight per frond (as % of control) ●
Dry/fresh weight ratio (%) □
300
200
100
30
20
10
1
2
3
4
5
6
7
Time in ABA (days)

Figure 20

The effect of incubation time on the fresh and dry weight levels per frond of S. polyrrhiza (N) in 1×10^{-7} M ABA.

○ Fresh weight per frond as % control

● Dry weight per frond as % control

□ Dry/fresh weight ratio (%)

Actual levels:

Fresh weight = 2690 → 1987 µg. frond^{-1}

Dry weight = 210 → 317 µg. frond^{-1}

FIGURE 20

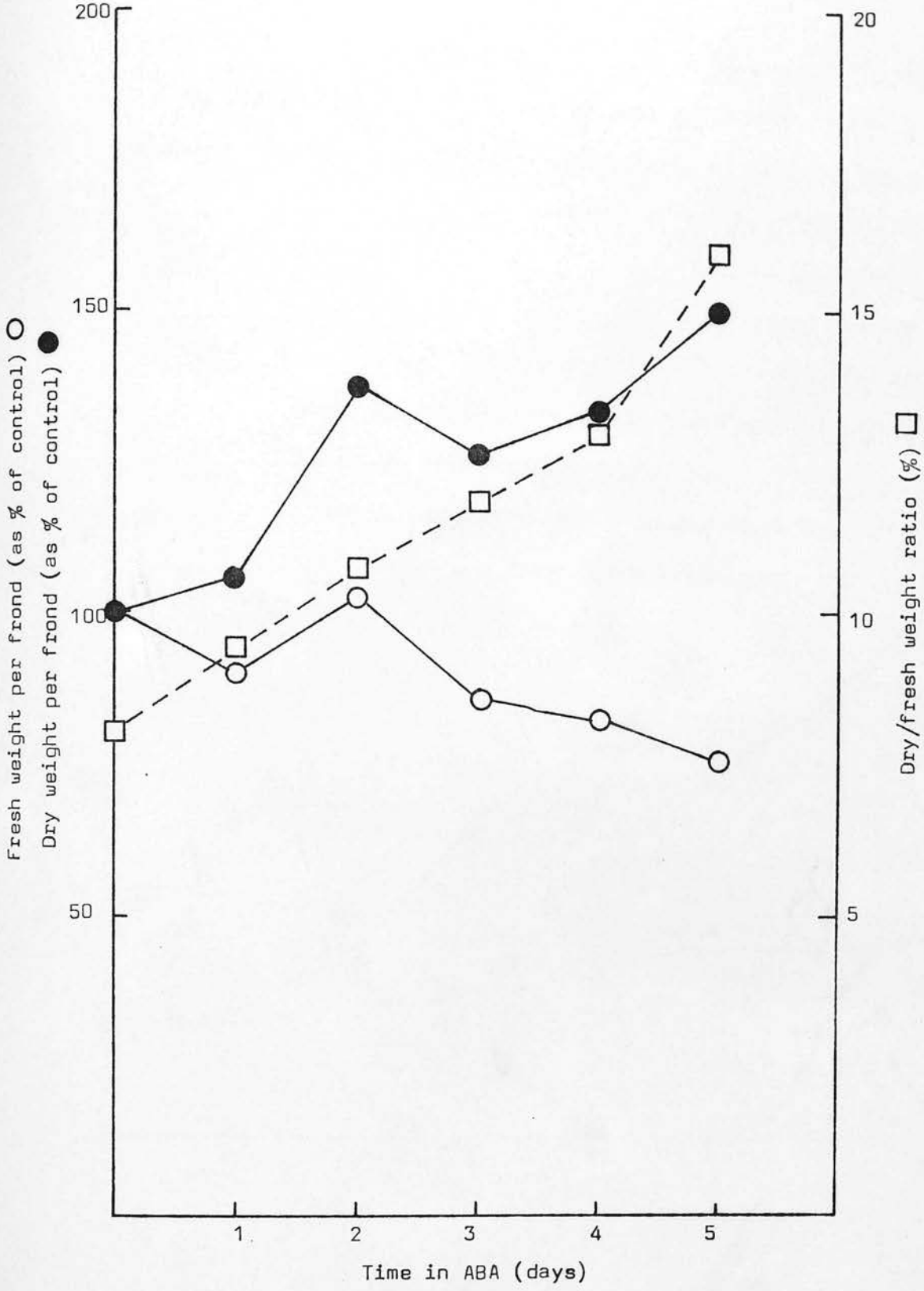

200
150
100
50
20
15
10
5
1
2
3
4
5
Time in ABA (days)
Fresh weight per frond (as % of control) ○
Dry weight per frond (as % of control) ●
Dry/fresh weight ratio (%) □

Figure 21

The effect of incubation time on the dry/fresh weight ratio of S. polyrrhiza (UC) in developing turions and the associated mother fronds incubated in 5×10^{-7}M ABA.

■ developing turions

□ mother fronds

Actual levels:

Developing turions fresh weight = 115 → 230 μg. frond^{-1}

" " dry weight = 20·3 → 66·9 μg. frond^{-1}

Mother fronds fresh weight = 1405 → 2633 μg. frond^{-1}

" " dry weight = 130 → 445 μg. frond^{-1}

FIGURE 21

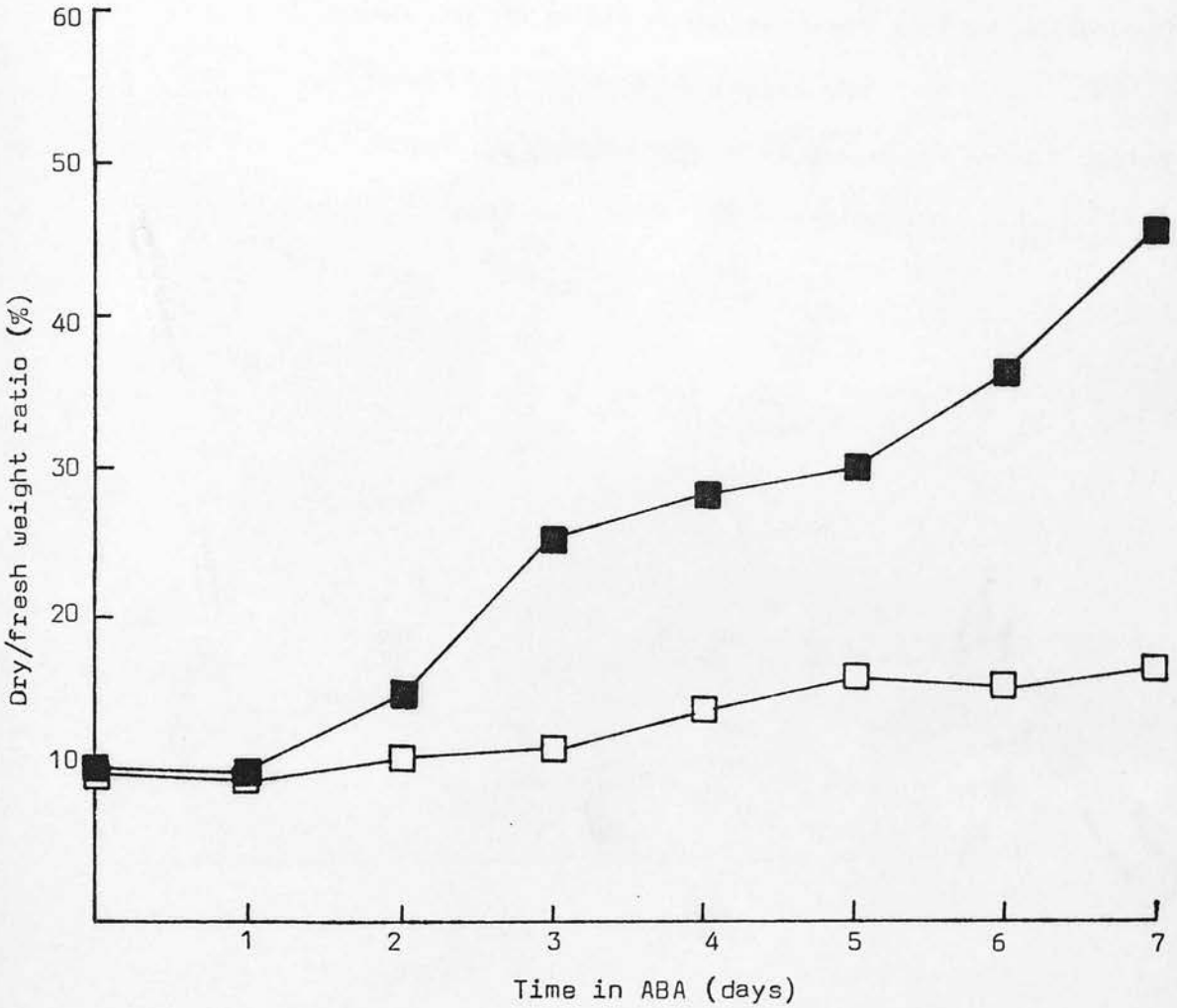

60
50
40
30
20
10
Dry/fresh weight ratio (%)
1
2
3
4
5
6
7
Time in ABA (days)

Figure 22

The effect of incubation time on the dry/fresh weight ratio of *S. polyrrhiza* (N) in developing turions and associated mother fronds incubated in 1×10^{-7} M ABA.

● developing turions

○ mother fronds

Actual levels:

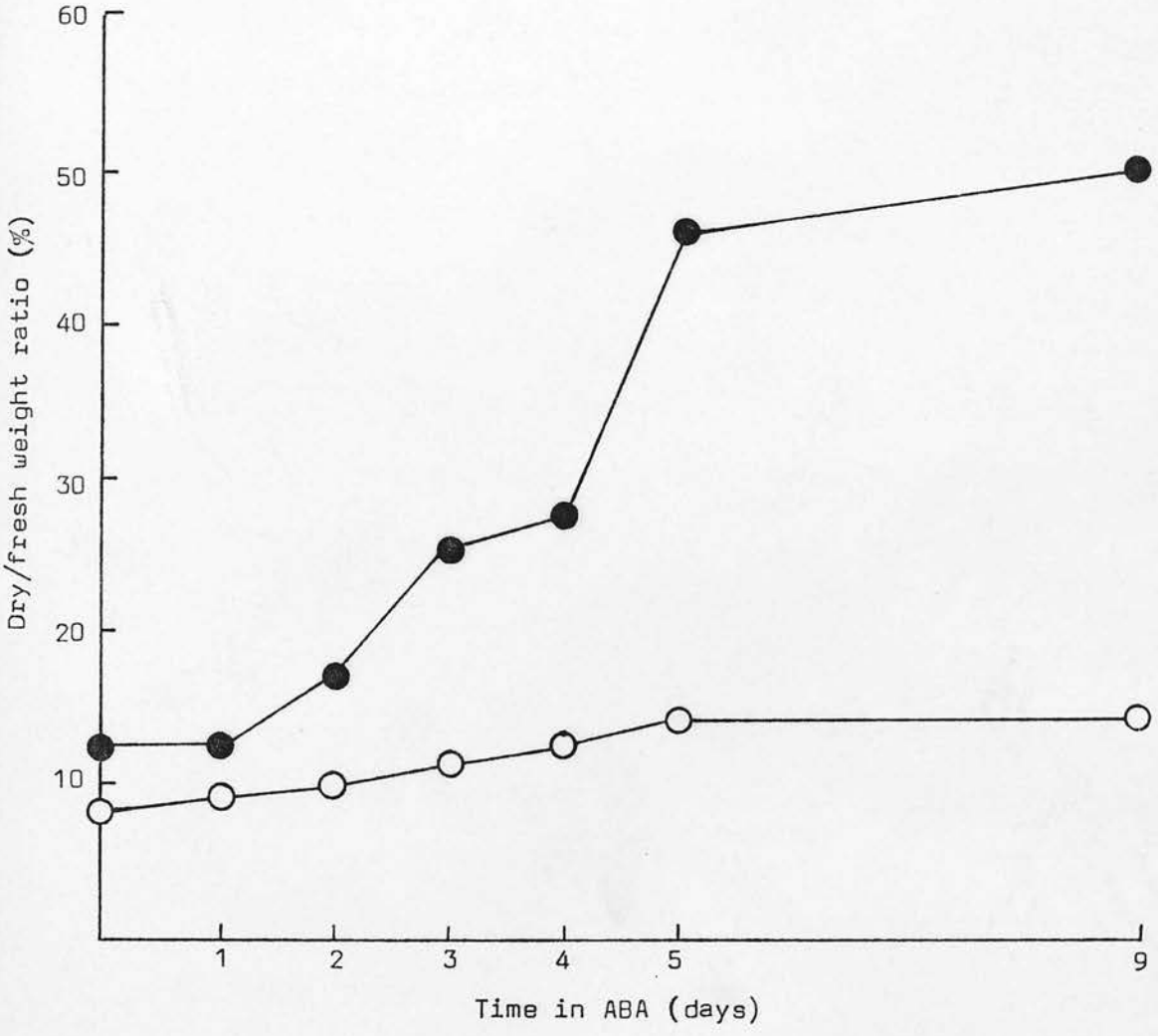
Developing turions fresh weight = 148 → 357 µg. frond^{-1}

" " dry weight = 19 → 181 µg. frond^{-1}

Mother fronds fresh weight = 2958 → 3543 µg. frond^{-1}

" " dry weight = 261 → 495 µg. frond^{-1}

FIGURE 22


60
50
40
30
20
10
Dry/fresh weight ratio (%)
1
2
3
4
5
9
Time in ABA (days)

shown that the starch content of S. polyrrhiza fronds also increases in the presence of ABA, however most of the starch accumulation at turion inducing concentrations of ABA occurs within the turion, rather than in the associated mother fronds. The time course of dry weight increase in S. polyrrhiza fronds at turion inducing concentrations of ABA is shown in figures 19 and 20. When the cultures were taken in entirety the fresh weight per frond decreased slightly, while the dry weight per frond increased dramatically. This increase was more noticeable in S. polyrrhiza (UC), which was at the time of the experiment entering one of its slow growing cycles and was producing very poor turions. The dry/fresh weight ratio increased from 7% to 22% after 7 days in S. polyrrhiza (UC) (Fig. 19), while fronds of S. polyrrhiza (N), which were growing well and produced large turions, increased their ratio from 8% to 16% after 5 days (Fig. 20).

The contribution of the developing turion to these changes in the dry/fresh weight ratio is shown in figures 21 and 22. It can be seen that most of the increase in the dry/fresh weight ratio of the fronds during incubation in ABA is found in the developing turions and not in the associated mother fronds. This effect was most pronounced in S. polyrrhiza (N), which produced large starch filled turions, and where the mother tissue did not accumulate large starch grains as confirmed by ultrastructural analysis. The turions of S. polyrrhiza (UC) were at this time more like miniature vegetative fronds, and although they were turion-shaped and heavily loaded with starch, starch grains had also accumulated in the mother fronds.

In S. polyrrhiza (N) the fresh and dry weight per frond of

developing turions and associated mother fronds is shown separately in order to show the massive dry weight increase in the developing turion (~10 fold) (Fig. 23). Although S. polyrrhiza (N) showed a slight decrease in fresh weight per frond when the culture was taken in entirety for measurement due to the development of turions (Fig. 20), when the developing turions were dissected from the tissue, neither the developing turions nor the associated mother fronds showed any decrease in fresh weight at this turion inducing concentration of ABA. The developing turions increased their fresh weight throughout the period of incubation in ABA, since the developmental stages dissected at day 0 were $\leqslant$0·7 mm long, whilst when fully developed at day 9 they were $\leqslant$2·1 mm long. Although all stages of developing turion were dissected for analysis at day 9, the average size of the fronds harvested was of course greater than the average size of those harvested before the addition of ABA.

Whether the starch accumulation found in S. polyrrhiza is due to an initial profound effect of ABA on carbohydrate metabolism, or is subsequent to an effect of ABA on growth is difficult to resolve. McCombs and Ralph (1972) reported increased starch content in S. oligorrhiza as a consequence of growth inhibition due to placing the fronds in the dark, while in L. minor growth inhibiting concentrations of 6-benzyladenine resulted in increased starch content, over and above that expected from growth inhibition alone (Tasseron-de-Jong and Veldstra, 1971). Both 10^{-7} M and 10^{-6} M ABA inhibit growth of L. minor plantlets (McLaren and Smith, 1976), while no increase in starch content was found with the lower concentration. However while S. polyrrhiza may be more sensitive to

Figure 23

The effect of incubation time on the fresh and dry weight levels per frond in developing turions and the associated mother fronds in *S. polyrrhiza* (N) incubated in 1×10^{-7} M ABA.

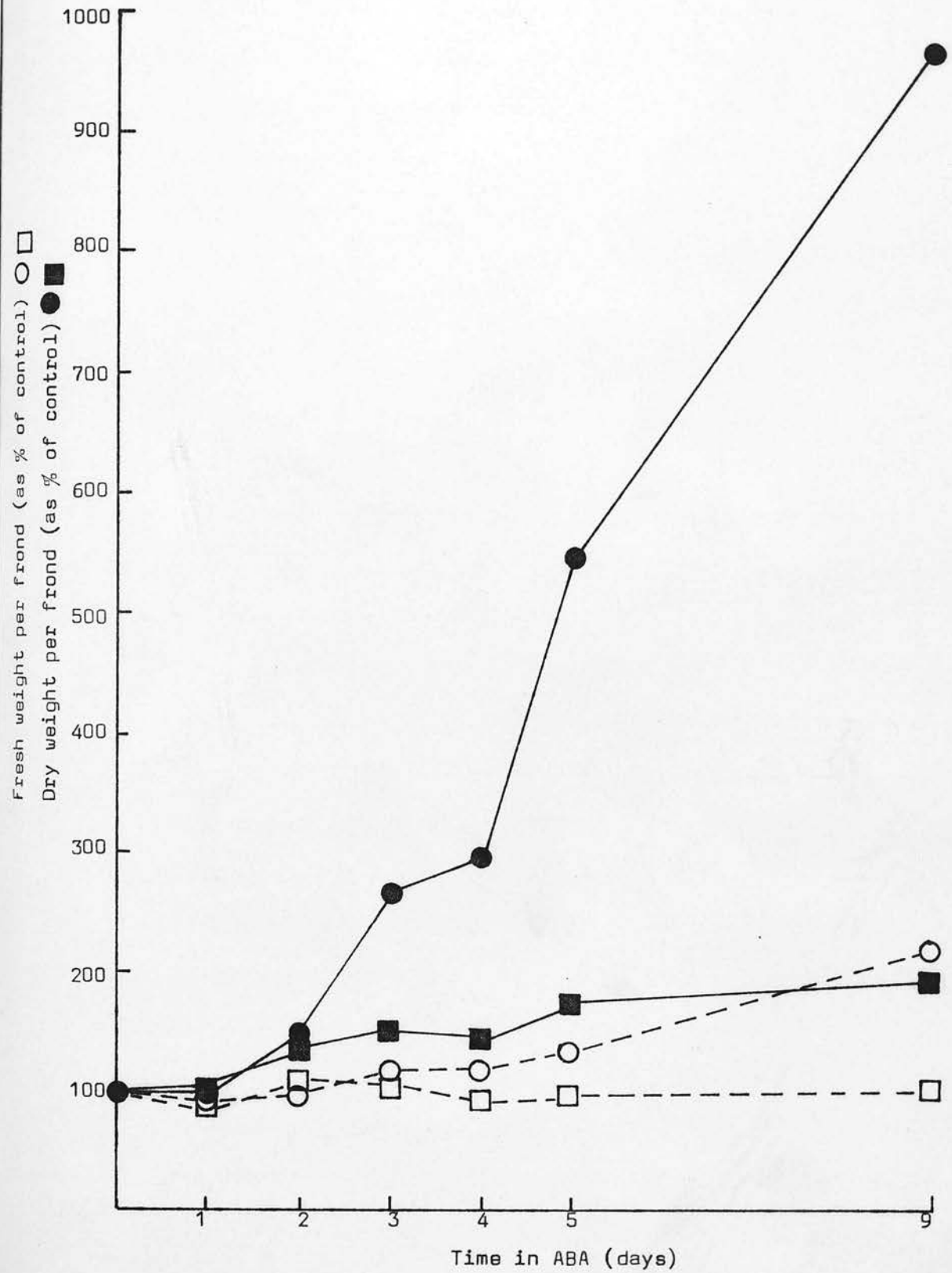
○ developing turions fresh weight per frond as % control

● developing turions dry weight per frond as % control

□ mother fronds fresh weight per frond as % control

■ mother fronds dry weight per frond as % control

FIGURE 23


Fresh weight per frond (as % of control) ○ □
Dry weight per frond (as % of control) ● ■
1000
900
800
700
600
500
400
300
200
100
1
2
3
4
5
9
Time in ABA (days)

ABA in this respect than L. minor which does not produce turions, any effect of ABA at low concentrations in younger perhaps more sensitive tissue would be masked by the presence of large amounts of mature and possibly insensitive tissue. In fact 10^{-7} M ABA had very little effect on the dry weight or starch accumulation in the majority of the fronds of S. polyrrhiza, but a very striking effect on the fronds destined to become turions.

McLaren and Smith (1976) also investigated starch accumulation induced by growth inhibition of L. minor by cyclohexamide, and found that a cyclohexamide concentration resulting in a similar rate of growth inhibition to 10^{-6} M ABA, resulted in starch increases of the order of one-third of that found with ABA.

Whilst there is little doubt that starch accumulation is characteristic of growth inhibition in general, it is not known whether ABA plays any additional role when it is the factor responsible for growth inhibition. Certainly environmentally or sugar induced turions contain high starch levels (Jacobs, 1947; Henssen, 1954), although ABA cannot be ruled out as the endogenous controlling factor in these turions. Recent evidence (Saks et al., 1980) indicates that turion forming cultures of S. polyrrhiza do in fact produce their own ABA, the time of appearance of the ABA and the rise in its concentration correlating with the onset of the process of turion formation. These authors suggest that ABA might be absorbed by young fronds where its synthesis might be low, thus causing the morphogenetic change characterised by turion formation instead of frond multiplication. However it is just as reasonable that their data is also consistent with a change in the sensitivity

of the cells of the frond throughout its development, rather than any differential synthesis of ABA in the course of maturity.

Any altered sensitivity to ABA might explain the difference in starch accumulation between mature and immature tissue, and evidence for differential sensitivity of cells to ABA even within a frond is seen during the formation of semi-turions, and even more strikingly in the absence of starch grains in the vascular tissue of the turion (sections 3.4.2 and 3.4.3).

It seems likely from the results of other workers in conjunction with the ultrastructural findings reported here that the increase in the dry/fresh weight ratio of the developing turion is due primarily to an increase in starch accumulation. Whilst it is felt that a net gain in starch is probably the major contributing factor to this increase, the levels of other important metabolites were also measured at turion inducing concentrations of ABA.

4.2 SUGAR LEVELS DURING TURION FORMATION

Treatment of fronds of S. polyrrhiza (N) with 10^{-7} M ABA resulted in a large increase in the level of sucrose, free glucose and free fructose (Fig. 24). Control fronds were found to have no measurable level of free fructose. Only after 3 days in ABA was free fructose detectable and this level increased from 2·2 µg. frond^{-1} to 3·7 µg. frond^{-1} over the following 2 days (Fig. 25). Glucose was present in fronds at very low levels, but increased from 105 ng. frond^{-1} in control fronds to 505 ng. frond^{-1} 5 days after ABA addition. The level of sucrose in the plantlets increased from 4·2 µg. frond^{-1} to 12·2 µg. frond^{-1} after 5 days in ABA.

Figure 24

The effect of incubation time on the sugar content of fronds of S. polyrrhiza (N) incubated in 1 x 10^{-7}M ABA. Sugars were extracted from the fronds with ethanol, evaporated to dryness and redissolved in water. Individual sugars were assayed in this water extract.

● sucrose

□ free fructose

○ free glucose

FIGURE 24

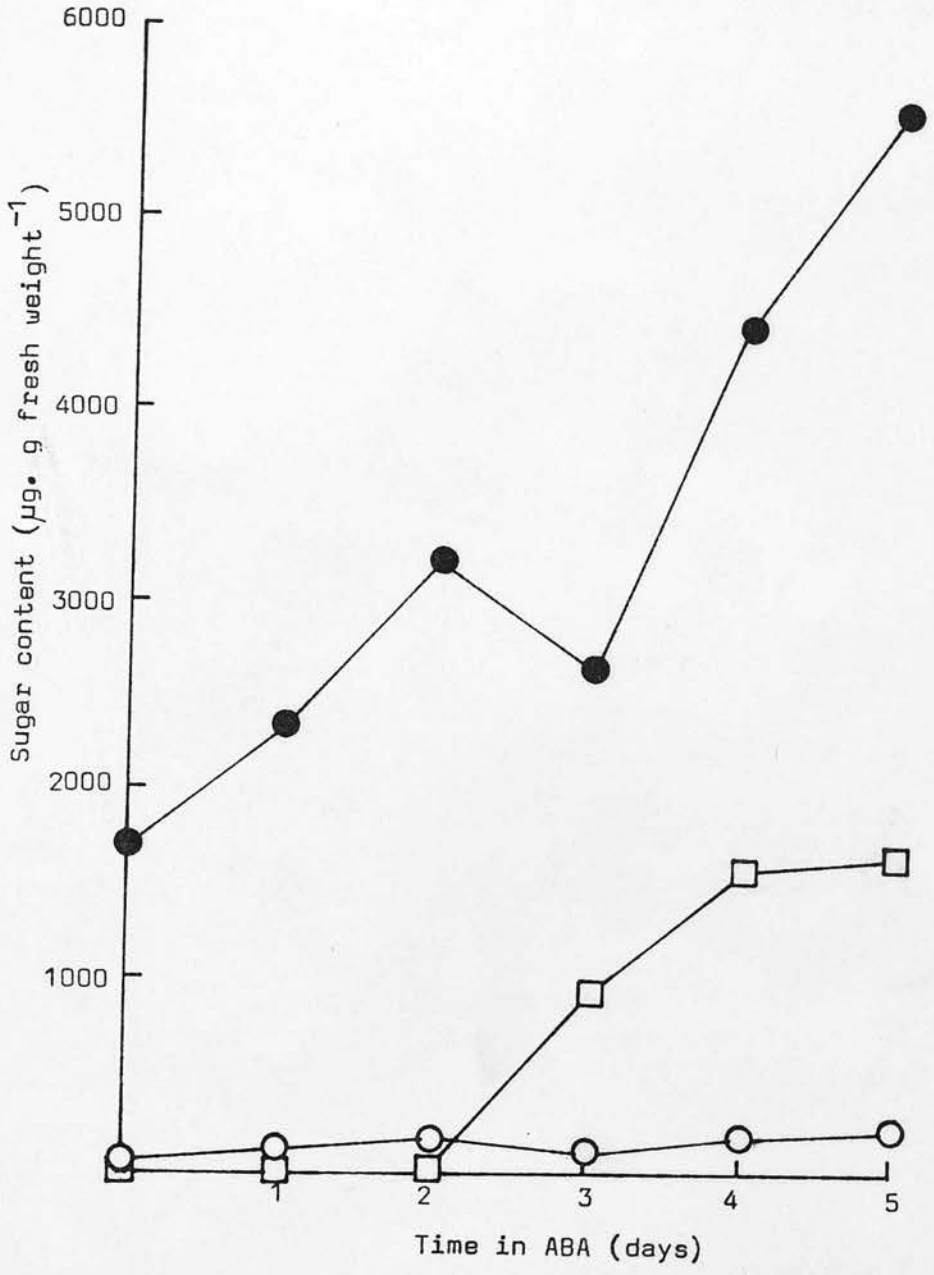

6000
5000
4000
3000
2000
1000
Sugar content (µg. g fresh weight^{-1})
1
2
3
4
5
Time in ABA (days)

Figure 25

The effect of incubation time on the sugar content of fronds of S. polyrrhiza (N) incubated in 1×10^{-7}M ABA. Sugar content is expressed on a per frond basis.

● sucrose

■ fructose

□ free fructose

○ free glucose

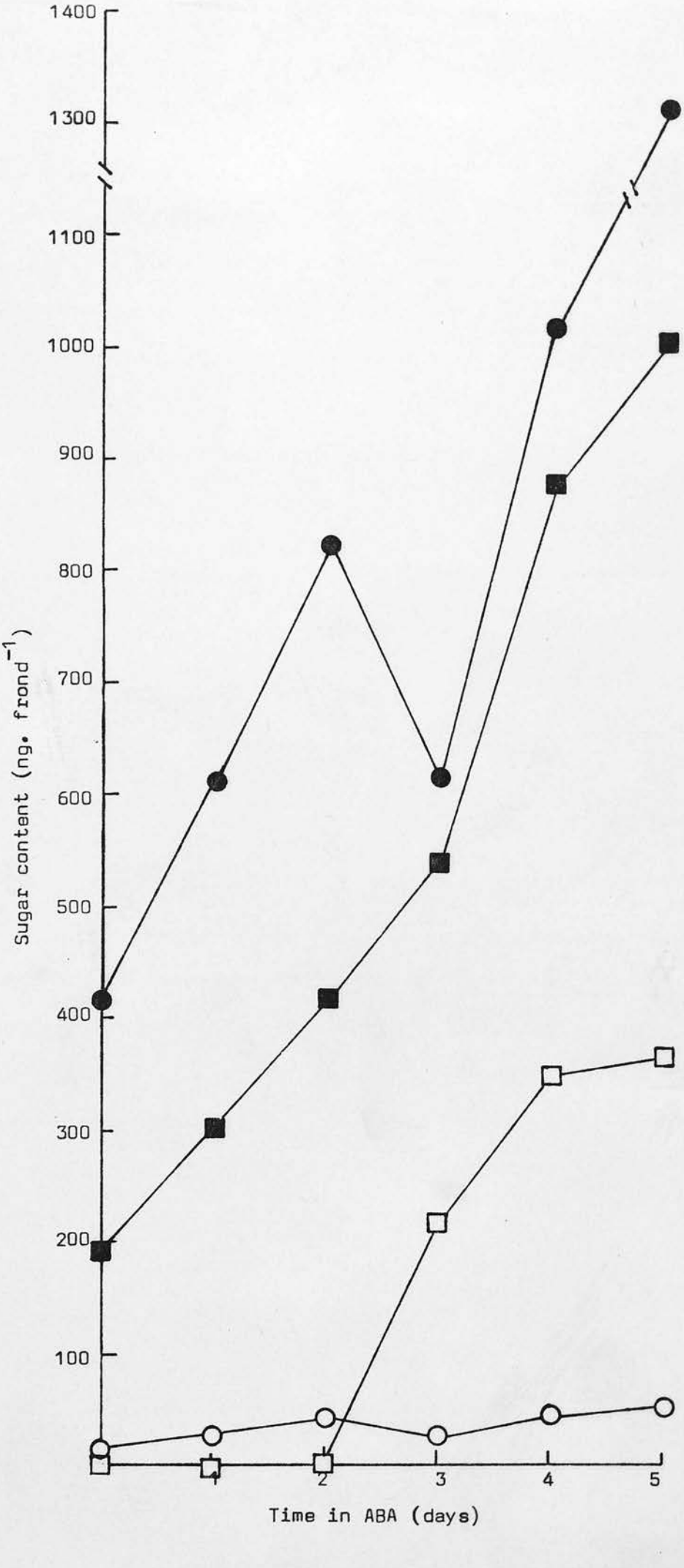

1400
1300
1100
1000
900
800
700
600
500
400
300
200
100
Sugar content (ng. frond^{-1})
1
2
3
4
5
Time in ABA (days)

FIGURE 25

When the sugar levels are represented as percentages of their control values (Fig. 26), it can be seen that within the first 2 days of ABA treatment, the level of glucose in the fronds rises the most rapidly, total fructose less so and sucrose least of all. Both glucose and sucrose levels appeared to drop between days 2 and 3, perhaps reflecting a period of large starch formation (cf. Fig. 22), or a period of maximum sugar utilisation during turion formation. At 3 days after ABA addition, the fronds contain free fructose, which increases the final total fructose content after 5 days to over 500% of the control value.

McLaren and Smith (1976) also reported that ABA at 10^{-6} M stimulated sugar levels in L. minor, and although they found that free fructose levels were higher than sucrose levels, and absolute sugar levels per gram fresh weight differed from those found in Spirodela polyrrhiza (N), stimulation of the sugar levels also occurred within 24 hours of ABA treatment. They also found that these increases could not be explained by growth inhibition alone.

ABA has been reported to reduce photosynthetic rate and RuBP carboxylase activity as measured by ^{14}C fixation methods (Mittelheuser and van Steveninck, 1971; Wellburn et al., 1973; Sankhla and Huber, 1974, 1975; Maillard-Sevhonkian and Pilet, 1978) and to reduce the Hill activity in isolated chloroplasts of L. minor but increase RuBP carboxylase activity (Bauer et al., 1976).

Carbohydrate assimilation, photosynthetic rate and respiration show a complex pattern which may be related to plant growth regulatory control of source-sink relationships (Neales and Incoll, 1968). Reduction of frond multiplication would effectively reduce

Figure 26

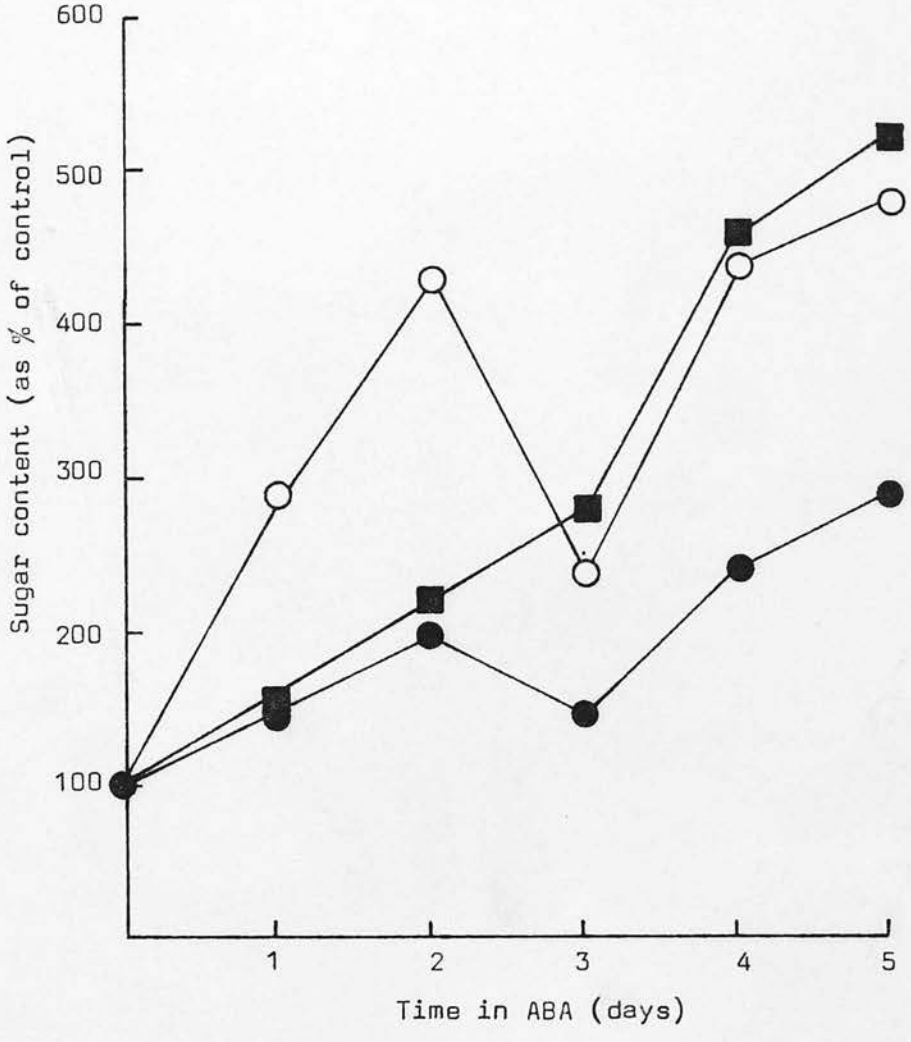
The effect of incubation time on the sugar content of S. polyrrhiza (N) incubated in 1 x 10^{-7}M ABA. Results expressed as % of control.

● sucrose

■ fructose

○ free glucose

FIGURE 26


600
500
400
300
200
100
Sugar content (as % of control)
1
2
3
4
5
Time in ABA (days)

the sink capacity of meristematic tissue and storage of assimilate in the carbohydrate pools would result (Mondal et al., 1978). In the case of S. polyrrhiza, not only is the frond multiplication rate reduced but the fronds that do develop (i.e. turions) are considerably smaller than normal vegetative fronds, resulting in the more pronounced starch and sugar accumulation seen in the turion. Although sugar levels were not measured separately in the developing turion (due to the variability of the assays used at low sugar concentrations), by analogy with dry weight accumulation one would suspect that the developing turions would also be more sensitive to ABA induced sugar accumulation than the mother fronds.

However growth regulator induced starch accumulation is reported to be greater than that expected from reducing the sink capacity of the meristem alone (Tasseron-de-Jong and Veldstra, 1971; McLaren and Smith, 1976; DeKock et al., 1978). Therefore ABA would seem also to have to act at a level which results in increased assimilate storage and consequential reduction in net photosynthetic rate.

McLaren and Smith (1976, 1977) found that the photosynthetic rate was higher in L. minor for the first 2 days of growth in ABA; but the sugar content was also high, suggesting that high levels of photosynthetically produced sugars do not have a direct feed-back effect on the photosynthetic rate during this period. Other evidence for this comes from the work of Natr et al. (1974) who found that high levels of exogenously applied glucose inhibited photosynthesis whereas carbohydrates accumulated by photosynthetic

assimilation had very little effect, suggesting that carbohydrate levels in the chloroplast and cytoplasm may have different effects on photosynthesis due to compartmentation. Similarly respiration is stimulated when L. minor is supplied with exogenous sucrose or glucose (McLaren and Smith, 1976), and when L. gibba is supplied with sucrose, fructose or glucose (Ullrich-Eberius et al., 1978), but the high sugar levels induced by ABA in neither case caused any concomitant rise in respiration (McLaren and Smith, 1976; Hartung et al., 1980). Hartung and co-workers concluded that since exogenously supplied glucose, fructose and sucrose increased respiration, while D-ribose had no effect, then the increase in sugar levels was probably due to ribose accumulation as a result of the inhibition of nucleic acid synthesis. While this might be so for L. gibba, the existence of separate pools of carbohydrate in the cell seems to have been discounted.

The ABA mediated increase in sugar levels in L. minor were found to be a result of decreased sugar utilisation rather than increased synthesis, and no evidence for ABA inhibition of starch degrading enzymes (e.g. α-amylase) was found to explain the starch accumulation (McLaren and Smith, 1976, 1977). Later inhibition of the photosynthetic rate (after 2 days) was not found to be associated with a reduced photochemical capacity, but presumably by a feed-back effect by the increased starch levels.

Only after 12 days growth in ABA could the decline in photosynthetic rate be accounted for by differences in the photochemical process, possibly due to starch grains within the chloroplasts having a shading effect on the thylakoids, or the starch

grains may be in fact disrupting the organisation of the granal stacks. Certainly in the turion of S. polyrrhiza the granal network is severely distorted and may explain the reduced photosynthetic rate found in the turion by Czopek (1967).

ABA has been reported to selectively alter the permeability of membranes (Mansfield and Jones, 1971) and the type of regulation discussed above could be brought about by ABA mediated changes in the chloroplast envelope, which would affect the balance of components between the chloroplast and cytoplasm (Heber, 1974).

It is interesting to note that sucrose, glucose and fructose have all been reported to induce turion formation in S. polyrrhiza (Jacobs, 1947; Czopek, 1963) and that sucrose stimulates starch accumulation in turions (Henssen, 1954). Sucrose has also been found to inhibit abscission in S. oligorrhiza (Witzum and Keren, 1978), and this is one of the characteristic effects of low concentrations of ABA (i.e. fronds tend to remain associated in plantlets).

Although starch and sugar accumulation is a characteristic effect of ABA and of turion formation in general, it may not be causal to turion formation, since no turions are formed under conditions of a high concentration of ABA which also result in high accumulated carbohydrate levels.

4.3 CHLOROPHYLL AND CAROTENOID LEVELS DURING TURION FORMATION

As the vegetative frond matures it shows a typical increase in its chlorophyll content whether the data are expressed on a frond or fresh weight basis (Fig. 27). The developing turion also shows a

Figure 27

The effect of incubation time on the chlorophyll content of developing turions and associated mother fronds incubated in 1×10^{-7} M ABA; and the chlorophyll content during the development of the vegetative frond. Chlorophyll was extracted from the tissue with acetone and chlorophyll content determined by spectrophotometry.

● developing turions

○ mother fronds

developing vegetative fronds

Levels per gram fresh weight basis:

Developing turions = 522 → 1014 $\mu g. g^{-1}$ (1185 $\mu g. g^{-1}$ fully mature turions, day 10)

Mother fronds = 754 → 904 $\mu g. g^{-1}$

Developing fronds = 623 → 1000 $\mu g. g^{-1}$

S. polyrrhiza (N)

FIGURE 27


4000
3000
2000
1000
Chlorophyll content (ng. frond^{-1})
1
2
3
4
5
6
7
10
Time in ABA (days) ● ○
Size of vegetative frond (≤mm)

similar increase during its development; and although the actual level of chlorophyll in the turion is much lower due to its small size, on a fresh weight basis it contains slightly more chlorophyll than the fully mature mother frond.

In view of reports that photosynthesis is severely lowered in the turion of S. polyrrhiza (Czopek, 1967) and of Wolffia arrhiza (Godziemba-Czyz, 1969b), this was rather surprising since the chlorophyll content of tissue is usually closely associated with its photosynthetic activity (Maksymowych, 1973). However the turions of Wolffia arrhiza were also found to have higher chlorophyll levels than the vegetative fronds. It therefore appears that total chlorophyll synthesis is relatively unaltered whether a primordium follows the normal or the turion programme of development.

The pattern of carotenoid accumulation is very similar to that of chlorophyll except that the developing turion preferentially accumulates carotenoids (Fig. 28). This is seen more clearly in figure 29, where the chlorophyll/carotenoid ratio increases from 4•7 to 7•7 during the development of the vegetative frond, while the developing turion shows an initial rise in the ratio to 6•3 after 1 day in ABA, whereafter it decreases gradually back to 4•8. The final concentration of carotenoids in the mature turion expressed on a fresh weight basis is twice that of the mature vegetative frond.

Although the effects of ABA cannot be separated from the inherent changes occurring in the pigment system during turion formation, ABA increased the carotenoid and the chlorophyll levels of the associated mother fronds, but only after 7 days in ABA. This

Figure 28

The effect of incubation time on the carotenoid content of developing turions and associated mother fronds of S. polyrrhiza (N) incubated in 1 x 10^{-7}M ABA; and the carotenoid content during the development of the vegetative frond. Carotenoids were extracted from the tissue with acetone and determined spectrophotometrically.

● developing turions

○ mother fronds

developing vegetative fronds

Levels on a gram fresh weight basis:

Developing turion = 112 → 193 µg. g^{-1} (248 µg. g^{-1} in day 10 fully mature turion)

Mother fronds = 104 → 129 µg. g^{-1}

Developing fronds = 133 → 131 µg. g^{-1}

FIGURE 28

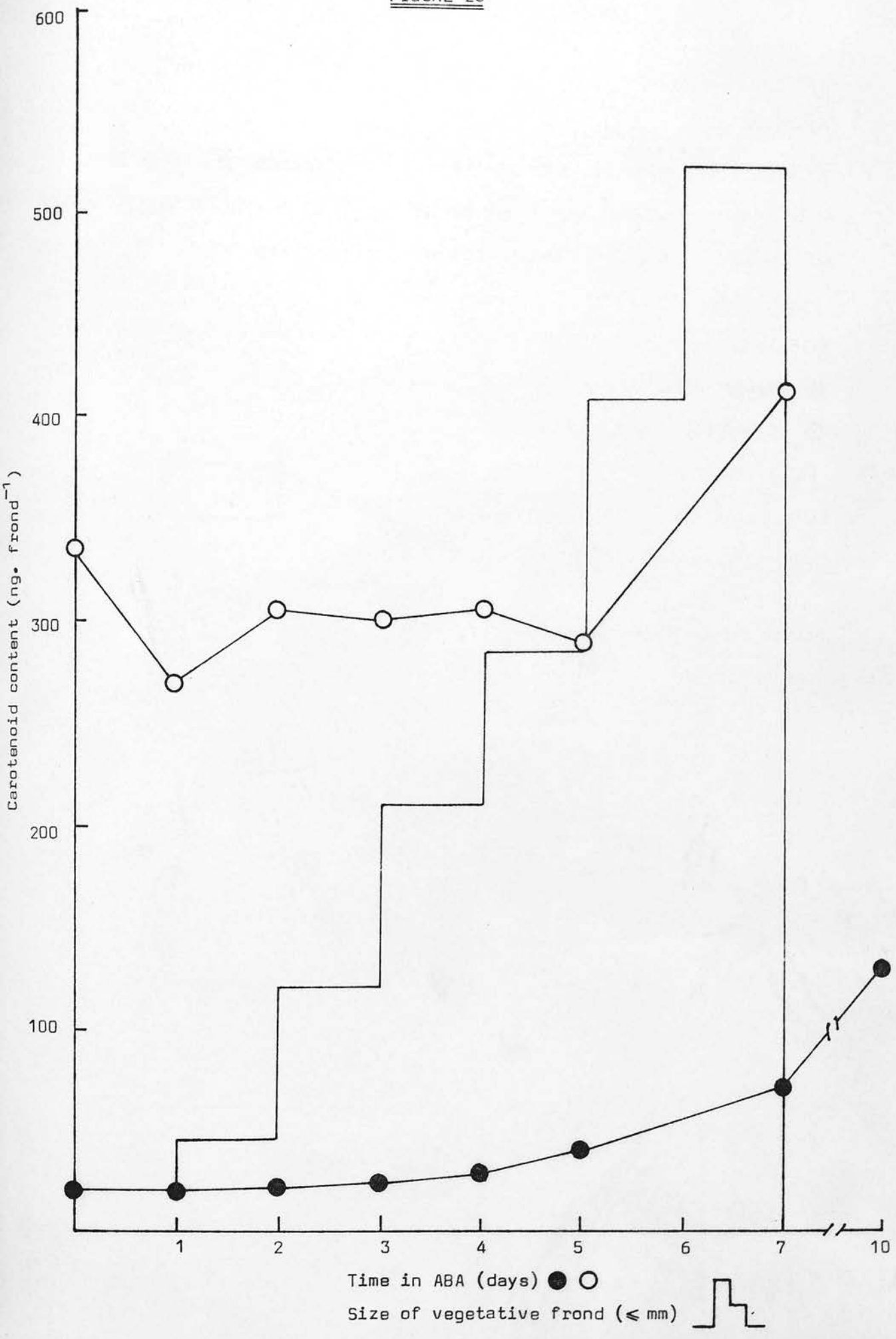

600
500
400
300
200
100
Carotenoid content (ng. frond^{-1})
1
2
3
4
5
6
7
10
Time in ABA (days) ● ○
Size of vegetative frond (≤ mm)

Figure 29

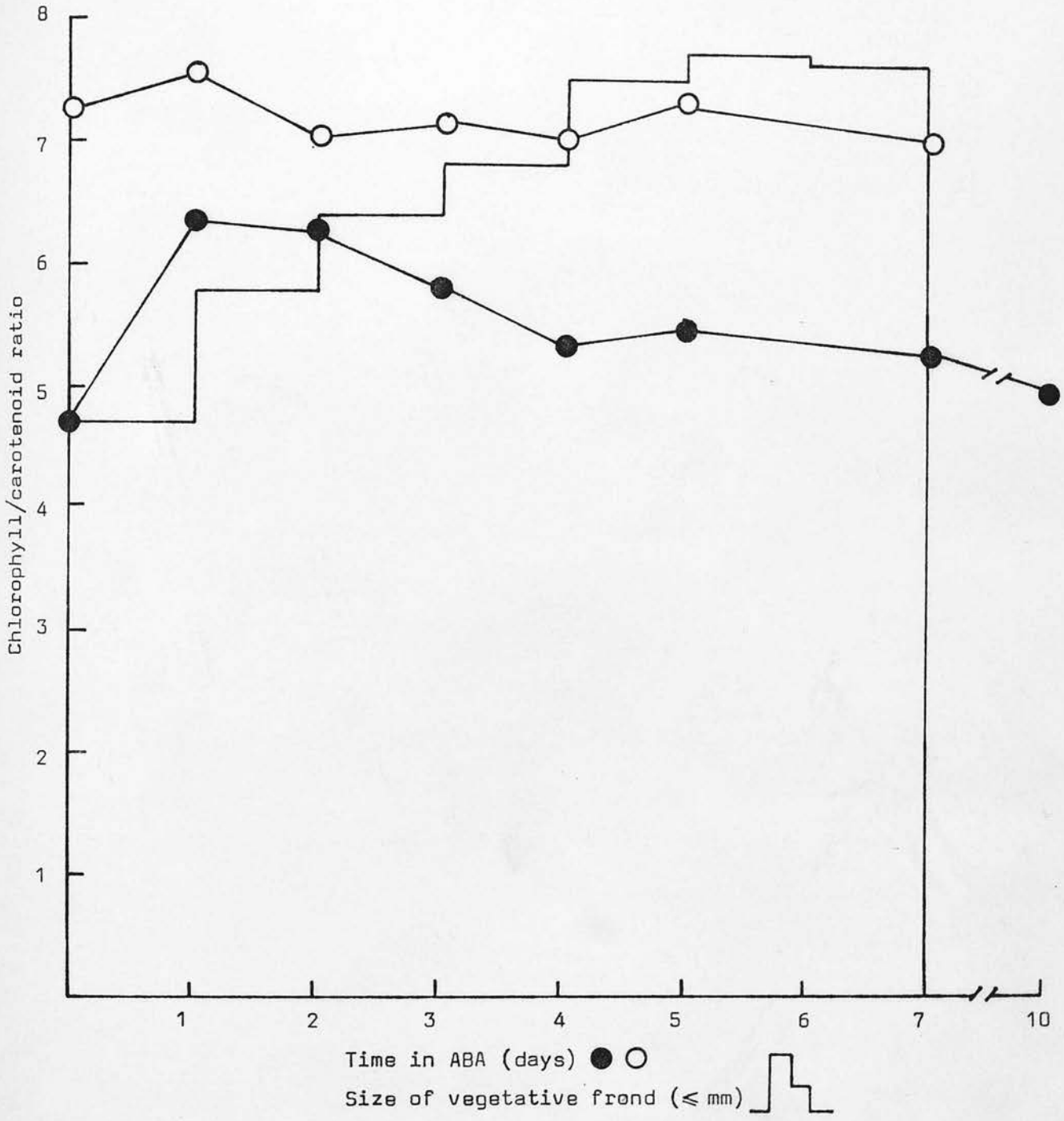
The effect of incubation time on the chlorophyll/carotenoid ratio in developing turions and associated mother fronds in 1×10^{-7} M ABA; and the ratio during the development of the vegetative frond of S. polyrrhiza (N).

● developing turions

○ mother fronds

developing vegetative fronds

FIGURE 29


Chlorophyll/carotenoid ratio
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
10
Time in ABA (days)
Size of vegetative frond (≤ mm)

effect was also reported by McLaren and Smith (1976) on whole plantlets of L. minor incubated at the same concentration of ABA (10^{-7} M) who found that carotenoid levels were increased by 40% and chlorophyll by 26%. The increase found in the mother fronds of S. polyrrhiza is slightly lower, with carotenoid levels stimulated by 24% and chlorophyll by 20%.

McLaren and Smith (1976) additionally found that the chlorophyll a/b ratio of the fronds increased from 3•2 to 4•4 at 10^{-7} M ABA, and even more dramatically at 10^{-6} M ABA to 7•2. ABA also had this effect on the mother fronds of S. polyrrhiza, where the ratio rose from 2•9 to 3•3 after 7 days in ABA (Fig. 30). The developing turions however, showed the opposite effect with the ratio declining from 3•2 to 2•2 during their development in ABA. This effect was in fact shown during the normal development of the vegetative frond although to a lesser extent, with an a/b ratio of 2•9 in the fully mature vegetative frond.

In systems where chlorophyll is accumulating rapidly as in the illumination of etiolated leaf segments, ABA has been found to inhibit chlorophyll accumulation (Poulson and Beevers, 1970; Duysen and Freeman, 1976). It is interesting to note that in etiolated wheat leaf segments, although ABA inhibited the light induced accumulation of chlorophyll, the chlorophyll a/b ratio increased in a manner similar to the effect of water stress on pigment accumulation; a phenomenon which is often accompanied by high concentrations of ABA (Wright and Hiron, 1970; Loveys and Kriedemann, 1973; Giles et al., 1974). It is evident that the extent to which pigment synthesis is disturbed by ABA depends upon the state of maturity of the material

Figure 30

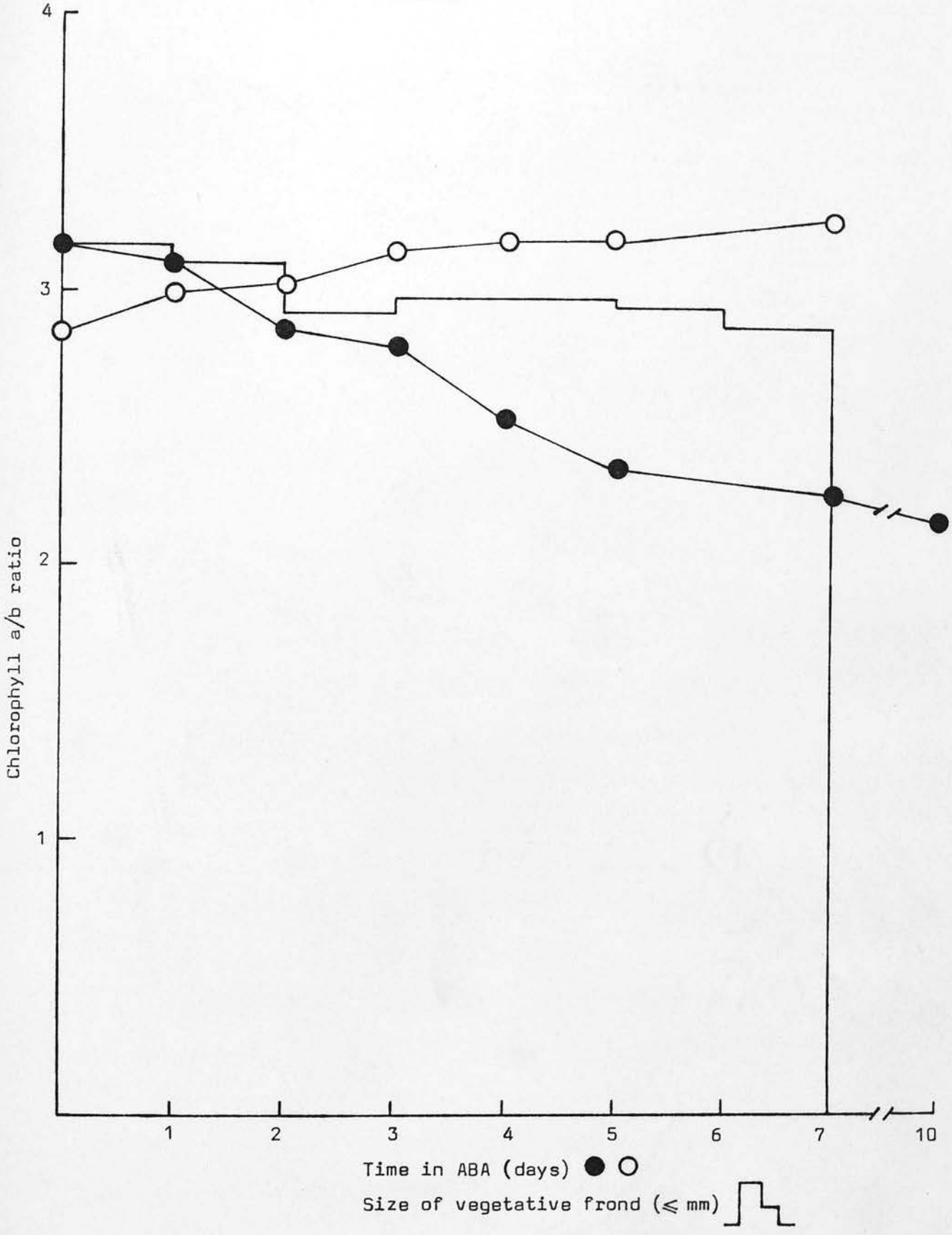
The effect of incubation time on the chlorophyll a/b ratio in the developing turion and associated mother fronds incubated in 1×10^{-7} M ABA; and the ratio during the development of the vegetative frond of S. polyrrhiza (N).

● developing turions

○ mother fronds

developing vegetative fronds

FIGURE 30


4
3
2
1
Chlorophyll a/b ratio
1
2
3
4
5
6
7
10
Time in ABA (days) ● ○
Size of vegetative frond (≤ mm)

under study.

Effects on chloroplast development and stability are also possible ways in which ABA might regulate overall levels of photosynthesis and, in seedlings of wheat and barley, Wellburn et al. (1973) showed that ABA inhibited plastid development both in vivo and in vitro during the early hours of greening. No adverse effects of ABA on the mature chloroplasts of S. polyrrhiza were found in this study or of L. minor by electron microscopy (Bauer et al., 1976). However some changes must occur because of the increased a/b ratio and carotenoid level found here and by McLaren and Smith (1976), and the increased starch grain size found by these workers; any or all of which might contribute to changes in the structural organisation of the organelle, and therefore to the reported changes in the rate of photosynthesis. The chloroplasts of the developing turion do however appear to be morphologically affected, and it seems likely that the low rate of photosynthesis reported is mainly due to the massive accumulation of starch within the chloroplast.

4.4 PROTEIN LEVELS DURING TURION FORMATION

Preliminary experiments with S. polyrrhiza (N) indicated no significant differences in the level of total protein in whole plantlets incubated in turion inducing concentrations of ABA, for up to 10 days (Fig. 31). However when fronds were dissected into developing turions and associated mother fronds, and their individual levels measured, noticeable trends became apparent (Figs. 32 and 33).

Figure 31

The effect of incubation time on the total protein content of fronds of S. polyrrhiza (UC) incubated in 5×10^{-7}M ABA. Protein was extracted from the fronds by homogenisation in NaOH, precipitation with TCA, solubilisation in NaOH and was determined by the Lowry method.

● $\mu g.\ frond^{-1}$

○ $mg.\ g^{-1}$

FIGURE 31

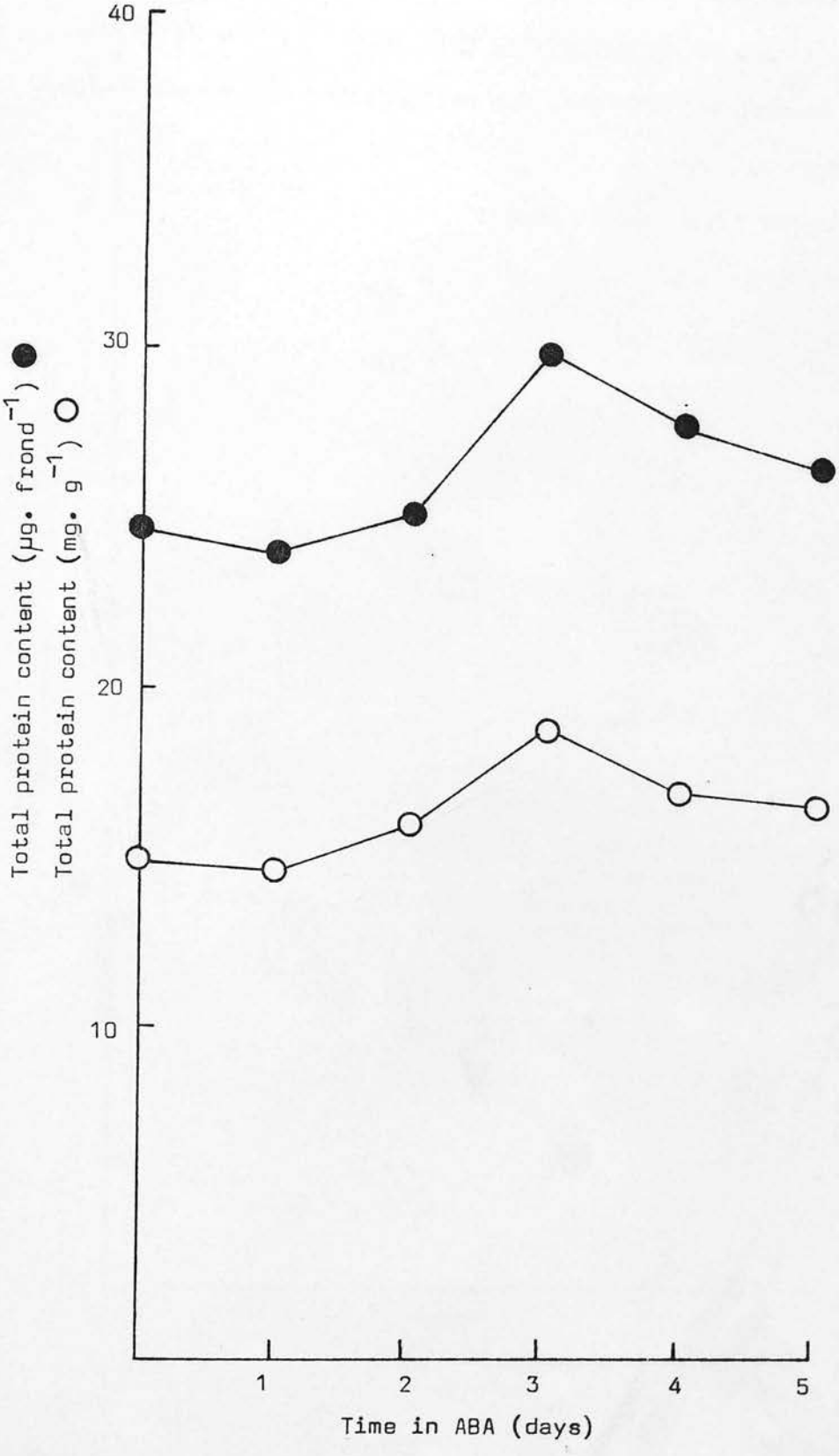

40
30
20
10
Total protein content (µg. frond^{-1}) ●
Total protein content (mg. g^{-1}) ○
1
2
3
4
5
Time in ABA (days)

Figure 32

The effect of time of incubation on the total protein content of developing turions of S. polyrrhiza (N) induced by 1 x 10^{-7}M ABA. Tissue was homogenised in NaOH, TCA precipitated and determined by the Lowry method.

Levels on a gram fresh weight basis:

26·9 → 26·8 mg. g^{-1}

FIGURE 32

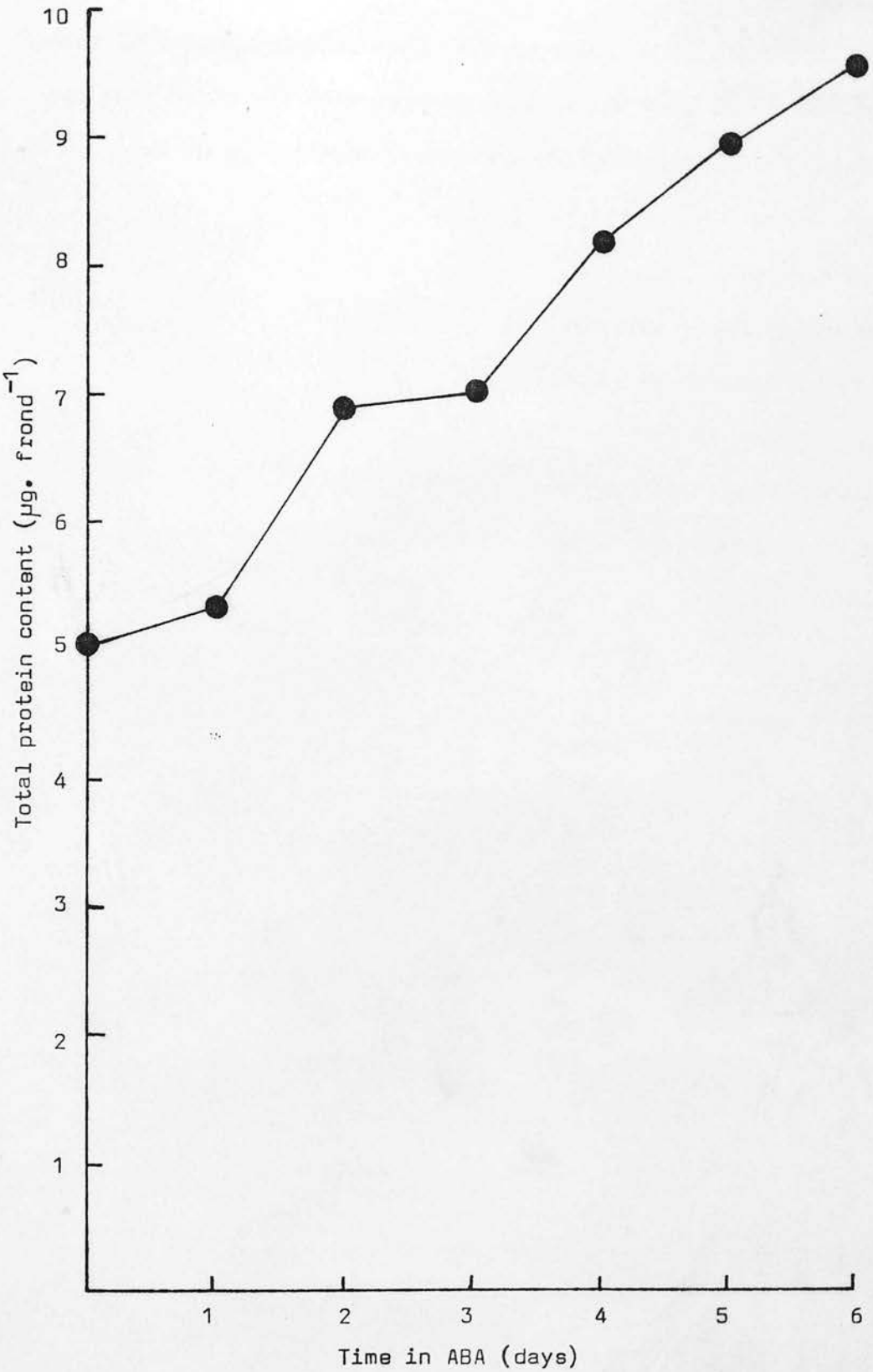

10
9
8
7
6
5
4
3
2
1
Total protein content (µg. frond^{-1})
1
2
3
4
5
6
Time in ABA (days)

Figure 33

The effect of incubation time on the total protein content of mother fronds associated with the turions incubated in 1 x 10^{-7}M ABA; and on total protein levels during the normal development of the vegetative frond of S. polyrrhiza (N).

O mother fronds

developing vegetative fronds

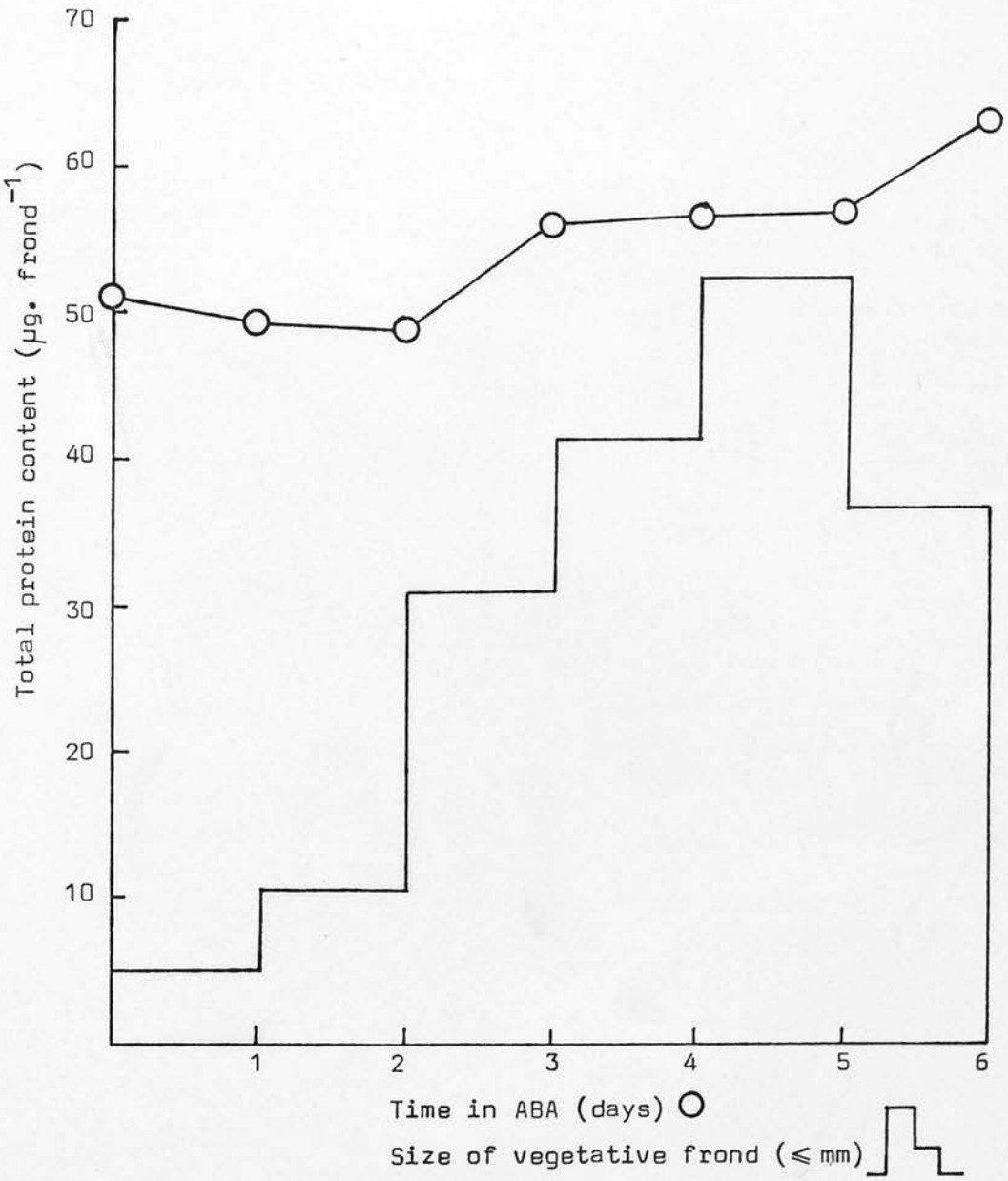
Levels on a gram fresh weight basis:

Mother fronds = 15·7 → 19·7 mg. g^{-1}

Developing vegetative fronds = 26·8 → 9·2 mg. g^{-1}

Tissue was homogenised in NaOH, TCA precipitated and protein was determined by the Lowry method.

FIGURE 33


70
60
50
40
30
20
10
Total protein content (µg. frond^{-1})
1
2
3
4
5
6
Time in ABA (days)
Size of vegetative frond (≤ mm)

Total protein in the developing turions increased with time of incubation in ABA when expressed on a frond basis (Fig. 32). This rise in protein content however, merely reflected the overall increase in average size of the developing turion, and is in fact less than would be expected from an examination of the protein content of different size classes of vegetative frond (Fig. 33). The level of protein in the developing vegetative frond increases with the average length of the fronds harvested up to fronds between 4 and 5 mm long. The largest fronds (5 - 6 mm) show a decline in the level of protein per frond, probably due to protein degradation associated with senescence. Fronds between 1 and 2 mm long have a slightly higher protein content than the approximately equivalent sized developing turions harvested after 6 days in ABA. The measurements made in section 4.1 on the fresh weight levels of the developing turion were used to give an approximation of the protein content on a fresh weight basis.

The rise in the protein level in the developing turion indeed corresponds to a constant level of protein of ~27 mg. g^{-1} during ABA incubation when so expressed on a fresh weight basis. The fresh weight of associated mother fronds however, does not alter during incubation in ABA (Fig. 23) and the rise of total protein in the mother fronds shown in figure 33 cannot be explained in the same way.

When soluble protein was examined separately from total protein, the results were even more interesting (Fig. 34). The soluble protein of the developing turion remains relatively constant throughout its development which reflects a sharp decline in soluble protein on a fresh weight basis from 10·4 mg. g^{-1} at day 0 to 4·9

Figure 34

The homogenate and soluble protein content of developing turions induced in 1 x 10^{-7} M ABA of S. polyrrhiza (N). Tissue was homogenised in grinding buffer and soluble protein separated by centrifugation. Protein was either acetone precipitated and determined by the Lowry method or used directly in the Bradford assay.

● homogenate protein

○ soluble protein

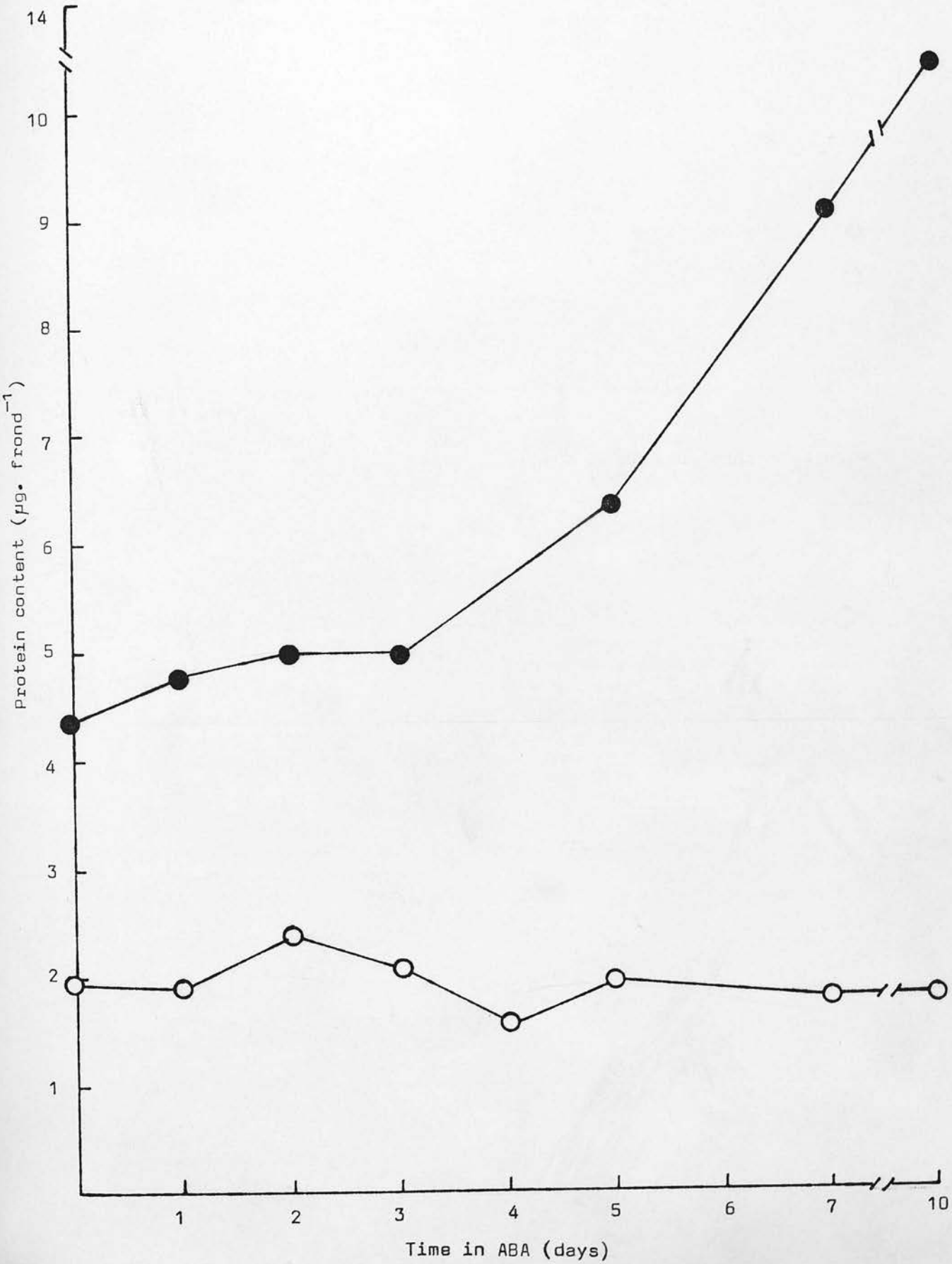
Levels on a gram fresh weight basis:

Homogenate protein = 23·2 → 25·5 mg. g^{-1} (25·8 mg. g^{-1})*

Soluble protein = 10·4 → 4·9 mg. g^{-1} (3·5 mg. g^{-1})*

* fully mature turions at day 10

FIGURE 34


14
10
9
8
7
6
5
4
3
2
1
Protein content (µg. frond^{-1})
1
2
3
4
5
6
7
10
Time in ABA (days)

mg. g^{-1} at day 7. Moreover the soluble protein of the developing vegetative frond rises during its development in line with the rise in total protein (Fig. 35). ABA effected an increase in the soluble protein in the mother fronds associated with the developing turions (Fig. 35).

The effect of ABA on the developing turions and the mother fronds therefore appears quite different; ABA increasing the level of both total and soluble protein in the mother fronds, while preferentially decreasing the amount of soluble protein in the developing turion.

Much of the rise in the total protein of the developing vegetative frond and the mother fronds associated with the developing turions was shown to be due to an increase in the soluble protein level, although the insoluble protein content also increased (Fig. 37). While the soluble protein content decreased during the development of the turion, this decline was not reflected in the insoluble protein content (Fig. 36), which increased during turion formation when expressed on either a frond or a fresh weight basis.

The decrease in soluble protein content during the development of the turion is thought to be inherent to the process of turion formation, since higher levels of ABA which do not induce the formation of turions, lead to an increase in the soluble protein content of fronds of the same average size as the developing turions at day 0, after 10 days in ABA. It therefore appears that tissue either insensitive to ABA with respect to turion formation i.e. mother fronds, or tissue sensitive but incubated in high non-turion forming concentrations of ABA, respond to ABA with an increase in

Figure 35

The homogenate and soluble protein contents of mother fronds incubated incubated in 1 x 10^{-7}M ABA; and the homogenate and soluble protein content of the developing vegetative frond of S. polyrrhiza (N).

■ mother fronds homogenate protein

□ mother fronds soluble protein

developing vegetative fronds homogenate protein

developing vegetative fronds soluble protein

Levels on a gram fresh weight basis:

Mother frond homogenate protein = 15•9 →27•3 mg. g^{-1}

" " soluble protein = 10•0 →17•9 mg. g^{-1}

Developing vegetative frond homogenate protein = 24•0 →20•4 mg. g^{-1}

" " " soluble protein = 11•2 →15•4 mg. g^{-1}

FIGURE 35

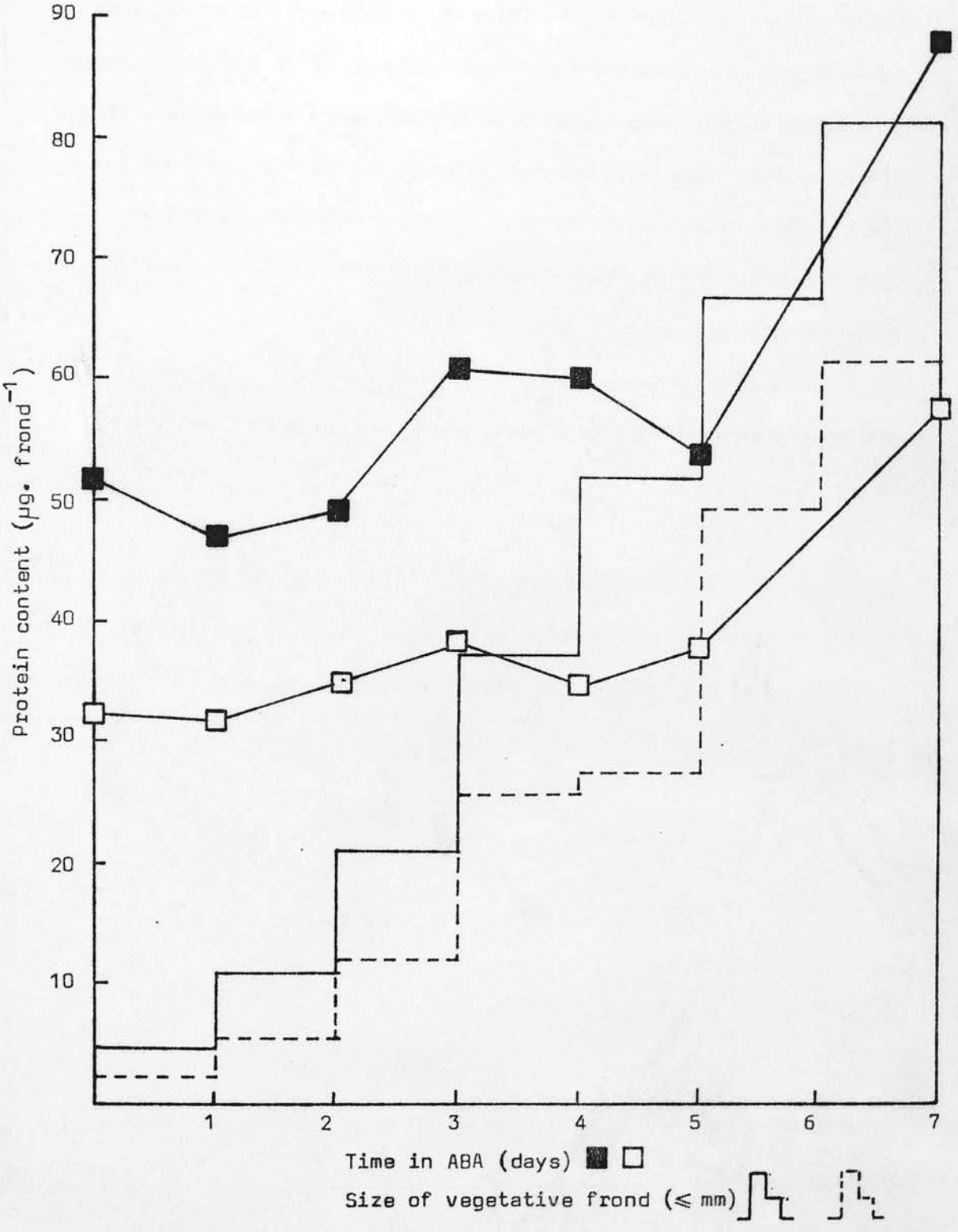

90
80
70
60
50
40
30
20
10
Protein content (µg. frond^{-1})
1
2
3
4
5
6
7
Time in ABA (days)
Size of vegetative frond (≤ mm)

Figure 36

The insoluble and soluble protein content during the development of the turion in 1 x 10^{-7}M ABA of S. polyrrhiza (N). Tissue was homogenised in grinding buffer and soluble and insoluble protein separated by centrifugation. Soluble protein was either acetone precipitated and determined by the Lowry method or directly assayed by the Bradford method. Insoluble protein was washed in grinding buffer, resuspended in the same, and either acetone precipitated for assay by the Lowry method or used directly for assay using the Bradford method (with and without solublisation with SDS buffer).

● insoluble protein

○ soluble protein

• insoluble protein*

o soluble protein*

Levels on a gram fresh weight basis:

Insoluble protein = 5·4 →7·7 mg. g^{-1} (8·0 mg. g^{-1})ǂ (9·0 mg. g^{-1})*

Soluble protein = 11·0 →5·3 mg. g^{-1} (2·6 mg. g^{-1})ǂ (12·3 mg. g^{-1})*

* fronds harvested (≤ 0·7 mm) from plantlets incubated at 1 x 10^{-5}M ABA

ǂ fully mature turions at day 10

FIGURE 36

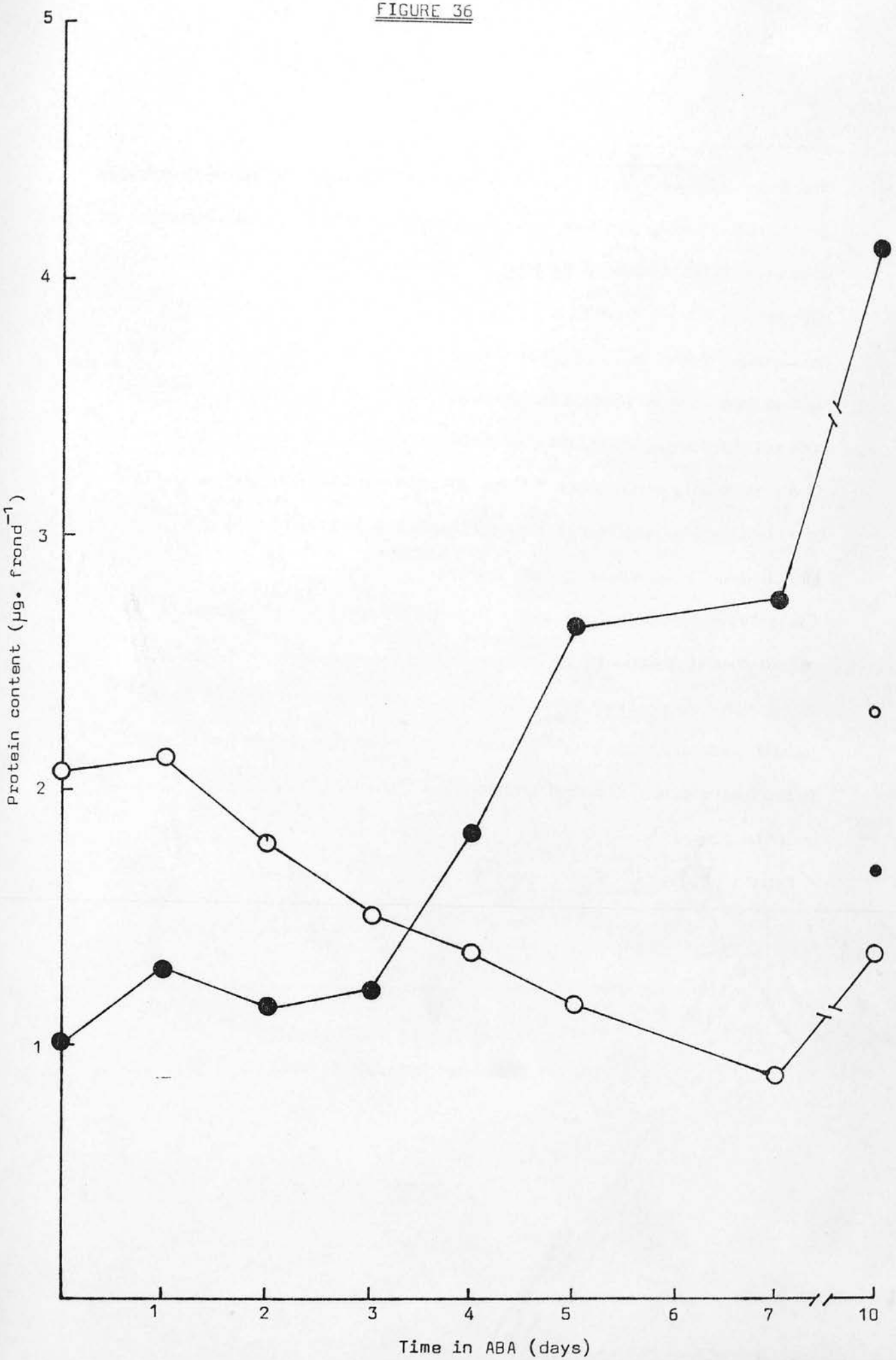


Figure 37

The insoluble and soluble protein content of mother fronds incubated in 1 x 10^{-7}M ABA; and the protein contents during the development of the vegetative frond of S. polyrrhiza (N).

■ mother frond soluble protein

▪ mother frond soluble protein*

□ mother fronds insoluble protein

▫ mother fronds insoluble protein*

developing vegetative fronds soluble protein

developing vegetative fronds insoluble protein

Levels on a gram fresh weight basis:

Mother frond soluble protein = 8·2 →11·7 mg. g^{-1} (13·9 mg. g^{-1})*

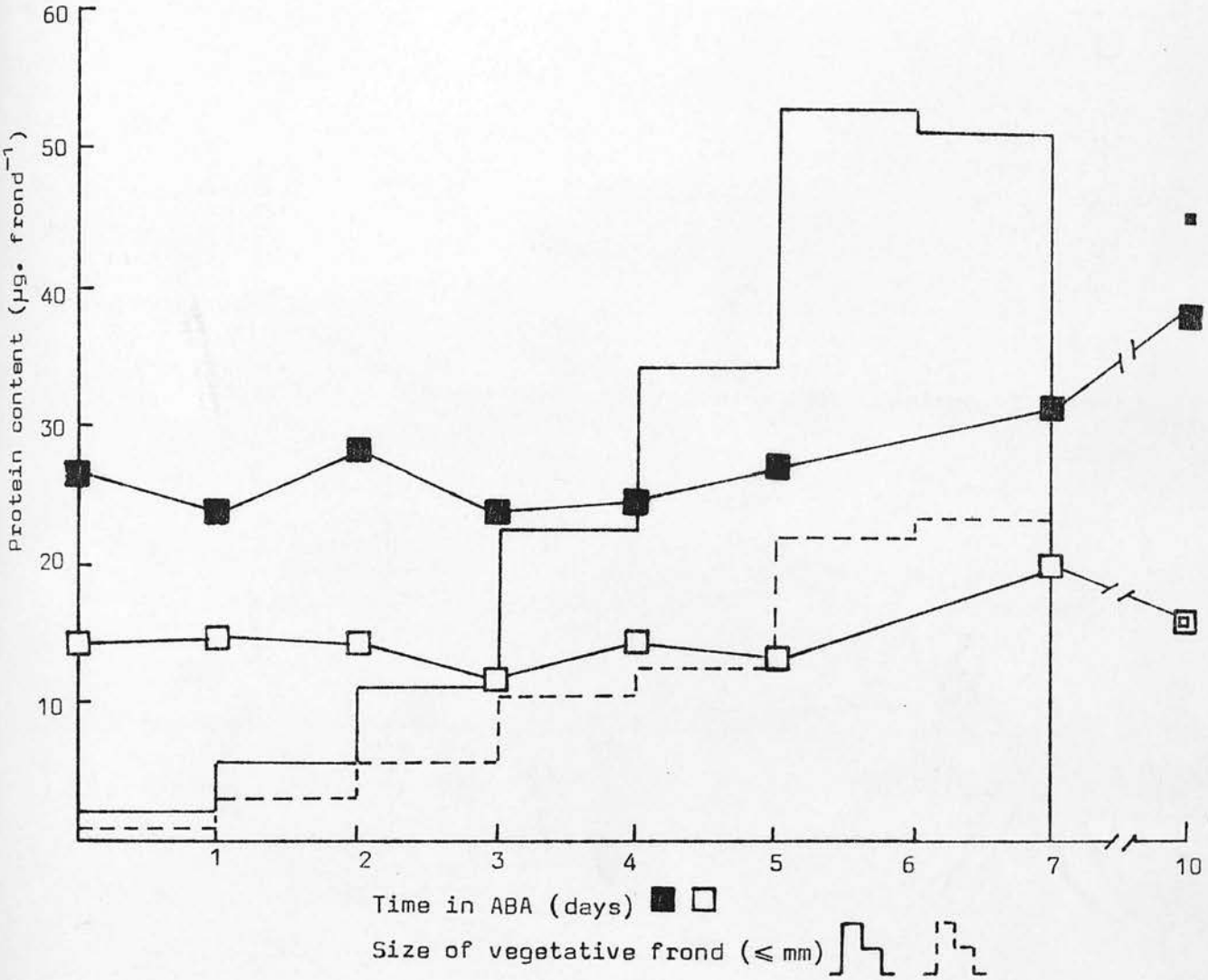
Mother frond insoluble protein = 4·3 →5·1 mg. g^{-1} (5·1 mg. g^{-1})*

Developing vegetative frond soluble protein = 10·6 →12·8 mg g^{-1}

Developing vegetative frond insoluble protein = 5·3 →5·8 mg. g^{-1}

* mother fronds incubated in 1 x 10^{-5}M ABA

FIGURE 37


60
50
40
30
20
10
Protein content (µg. frond^{-1})
1
2
3
4
5
6
7
10
Time in ABA (days)
Size of vegetative frond (≤ mm)

soluble protein level.

Although ABA induces turion formation, it only does so over a specific concentration range. The effect of ABA in lowering soluble protein levels appears to be specific to the process of turion formation, and indeed ABA induces the accumulation of soluble protein in tissue not involved in this morphogenesis.

The results obtained for the mother fronds of S. polyrrhiza, showing an increase in protein levels with incubation in ABA are rather surprising in view of other published data. Trewavas (1972b) found that 5×10^{-6} M ABA decreased total protein levels in L. minor due to decreased protein synthesis and enhanced protein degradation, while Andres and Smith (1976) reported no effect of 10^{-6} M ABA on the protein content of S. polyrrhiza, L. minor or L. paucicostata fronds, although they did find a profound effect on amino acid efflux from the tissue, all species showing enhanced efflux of amino acids into the medium after only 1 hour in ABA.

Since Fraction 1 protein represents a large proportion of the soluble protein of plant tissue (Ellis, 1979), the levels of this protein were investigated during turion formation by gel electrophoresis, to see if this protein followed the general pattern shown by the soluble protein as a whole. Fraction 1 protein also declined in amount during turion formation, and increased in all other tissues studied, thus following the general trend of the soluble protein. However the initial increase in Fraction 1 protein content of the developing turions seen after 1 day in ABA was not seen in the soluble protein generally. Although the fully developed turion after 10 days in ABA contained 66% of the soluble protein and

Figure 38

Fraction 1 protein content during the development of the turion and in associated mother fronds incubated in 1×10^{-7} M ABA; and the Fraction 1 protein content during the normal development of the vegetative frond. Fraction 1 protein was determined by non-denaturing gel electrophoresis.

● developing turions

○ mother fronds

developing vegetative fronds

Levels on a per gram basis:

developing turions = 6·2 → 2·8 mg. g^{-1} (1·3 mg. g^{-1})*

mother fronds = 7·1 → 10·7 mg. g^{-1}

developing vegetative fronds = 12·9 → 16·4 mg. g^{-1}

* fully mature turions at day 10

FIGURE 38


Fraction 1 protein content (µg. frond^{-1}) ●
2
1
Fraction 1 protein content (µg. frond^{-1}) ○
70
60
50
40
30
20
10
1
2
3
4
5
6
7
10
Time in ABA (days) ● ○
Size of vegetative frond (≤ mm)

of the Fraction 1 protein found before the addition of ABA (i.e. total decrease in protein was identical), Fraction 1 protein was not affected as quickly as the soluble protein as a whole (Fig. 38).

If the lowered protein levels during turion formation were due to a blanket decrease in the overall synthesis of soluble protein, then differential turnover rates between types of protein could account for these differences.

4.5 SPECIFIC CHANGES IN THE SOLUBLE PROTEINS DURING TURION FORMATION

Changes in the pattern of other soluble proteins were investigated by 2-dimensional polyacrylamide gel elctrophoresis. During the development of the turion several changes in the pattern of proteins can be observed (Fig. 39).

Firstly protein 4 (53,000 daltons; pI = 5·88) which is the major stained protein in the developing turion (and is most likely the large subunit of Fraction 1 protein (Ellis et al., 1977), decreases in abundance during turion formation, to become the 4th most abundant protein after 7 days in ABA. At the same time another protein 40 (52,000 daltons; pI = 5·06) increases from being a minor protein at day 0 to the most abundant protein after 7 days in ABA. Several other changes are apparent. The high molecular weight proteins at approximately 80,000 daltons disappear during turion formation, as well as many other minor species. However the most interesting feature during turion formation in these stained gels is the increase in the amounts of certain proteins, since there is a net overall decrease in the amount of soluble protein. Of these, the most noticeable are proteins 35, 45, 50 and 51 which increase in

Figure 39

Pattern of Coomassie blue stained proteins during the development of the turion of S. polyrrhiza (N) in 1 x 10^{-7} M ABA. Soluble protein was extracted from each developmental stage and loaded onto 2-dimensional polyacrylamide gels. Equal amounts of soluble protein were applied to each gel (50 µg).

a) day 0 (untreated)

b) day 1

c) day 2

d) day 3

e) day 4

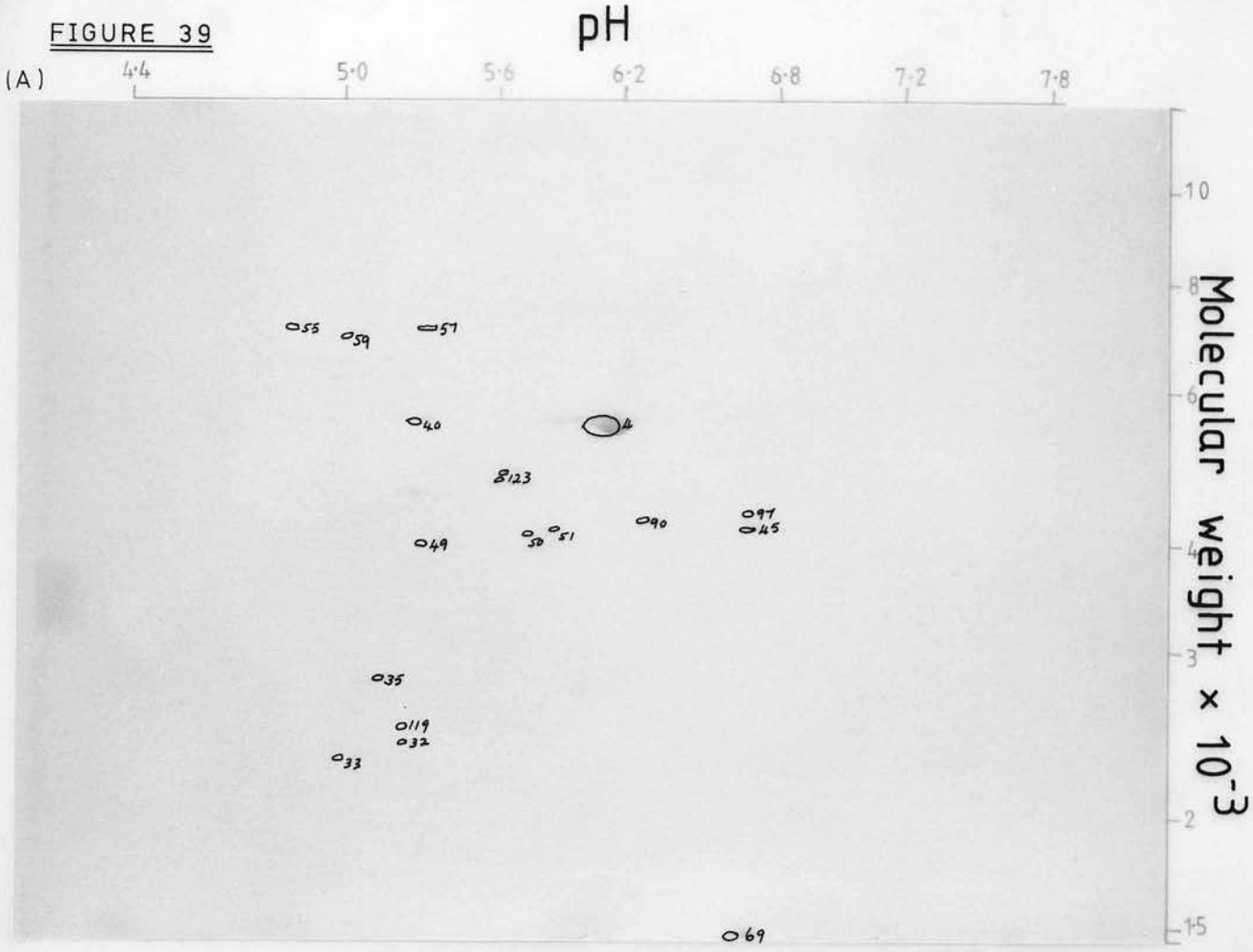
f) day 5

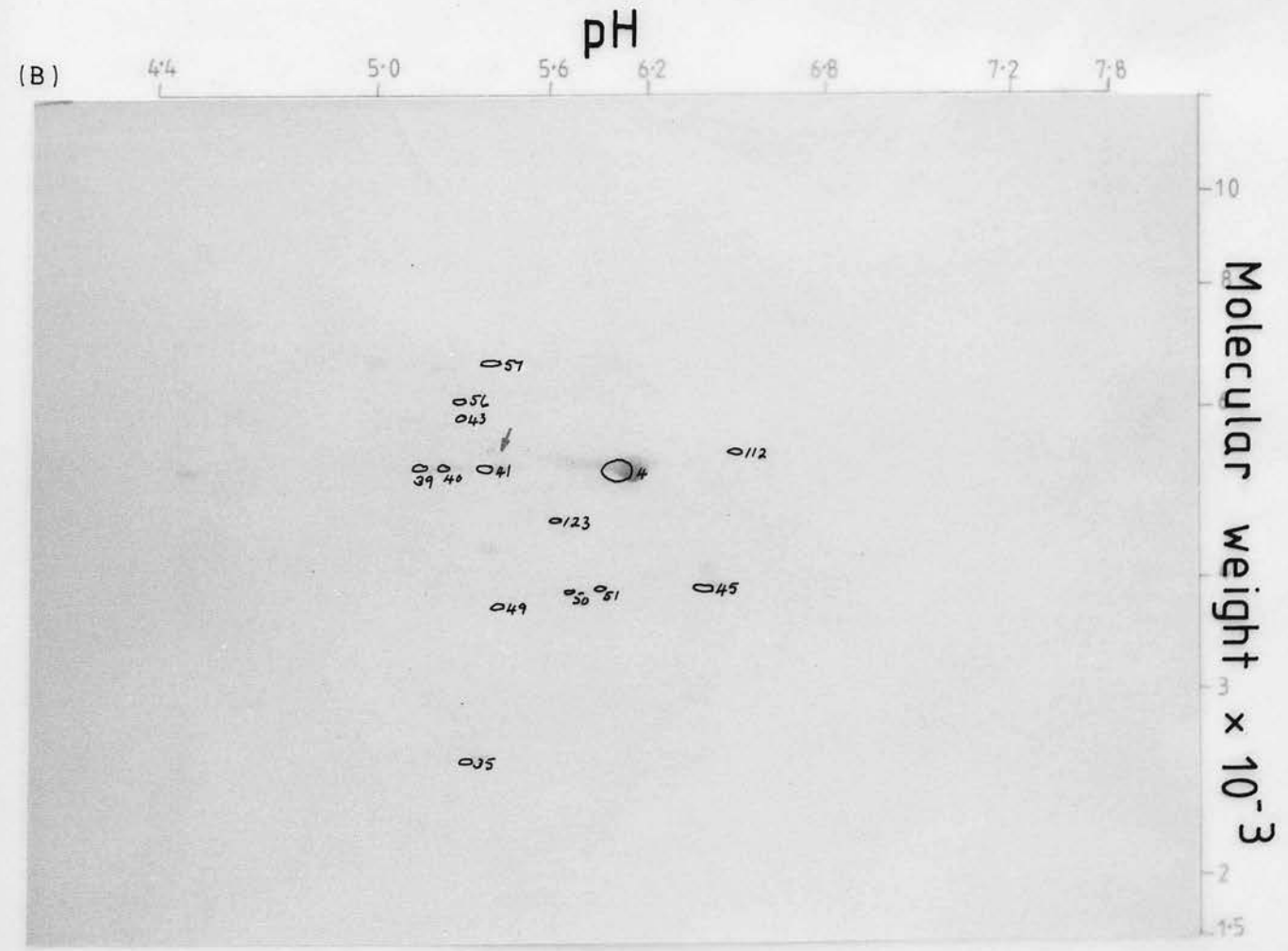
g) day 7

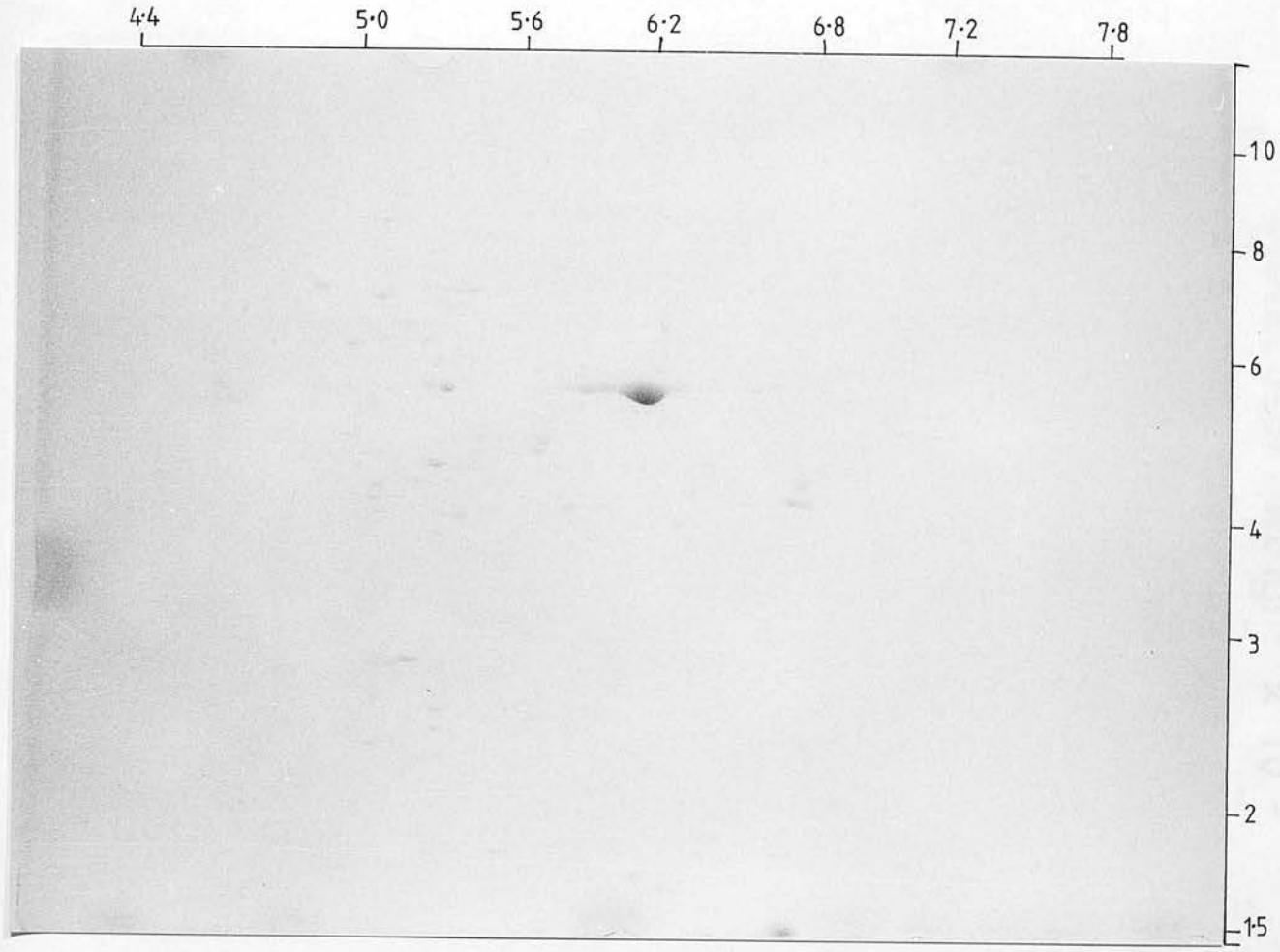
↘ indicates that protein has increased in amount from the day before

↖ indicates that protein has decreased in amount on the next day

FIGURE 39


(A)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10⁻³
10
8
6
4
3
2
1·5
55
59
57
40
4
123
90
97
45
49
50
51
35
119
32
33
69


(B)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10⁻³
10
8
3
2
1·5
57
56
43
112
39
40
41
4
123
45
49
50
51
35


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

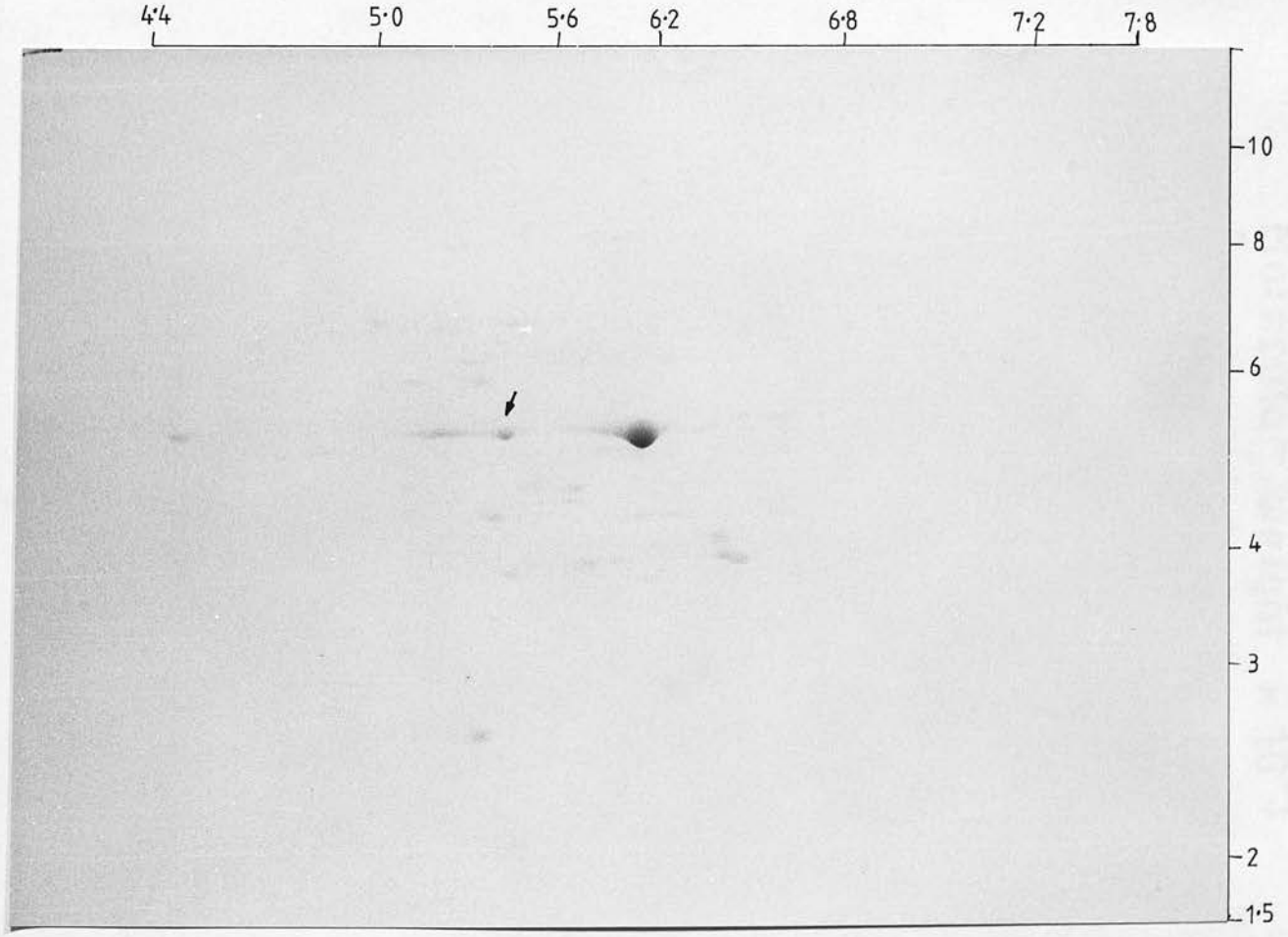
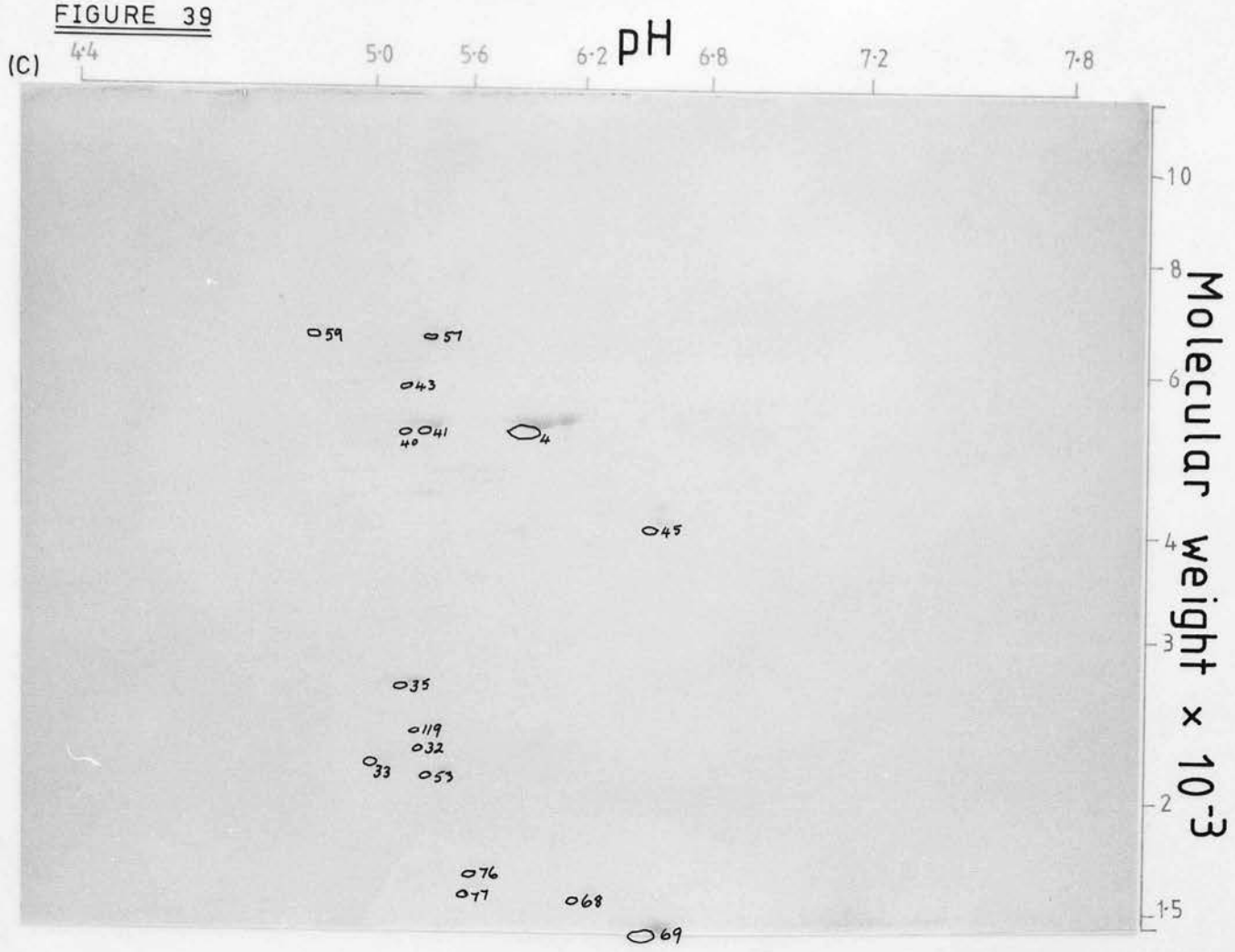
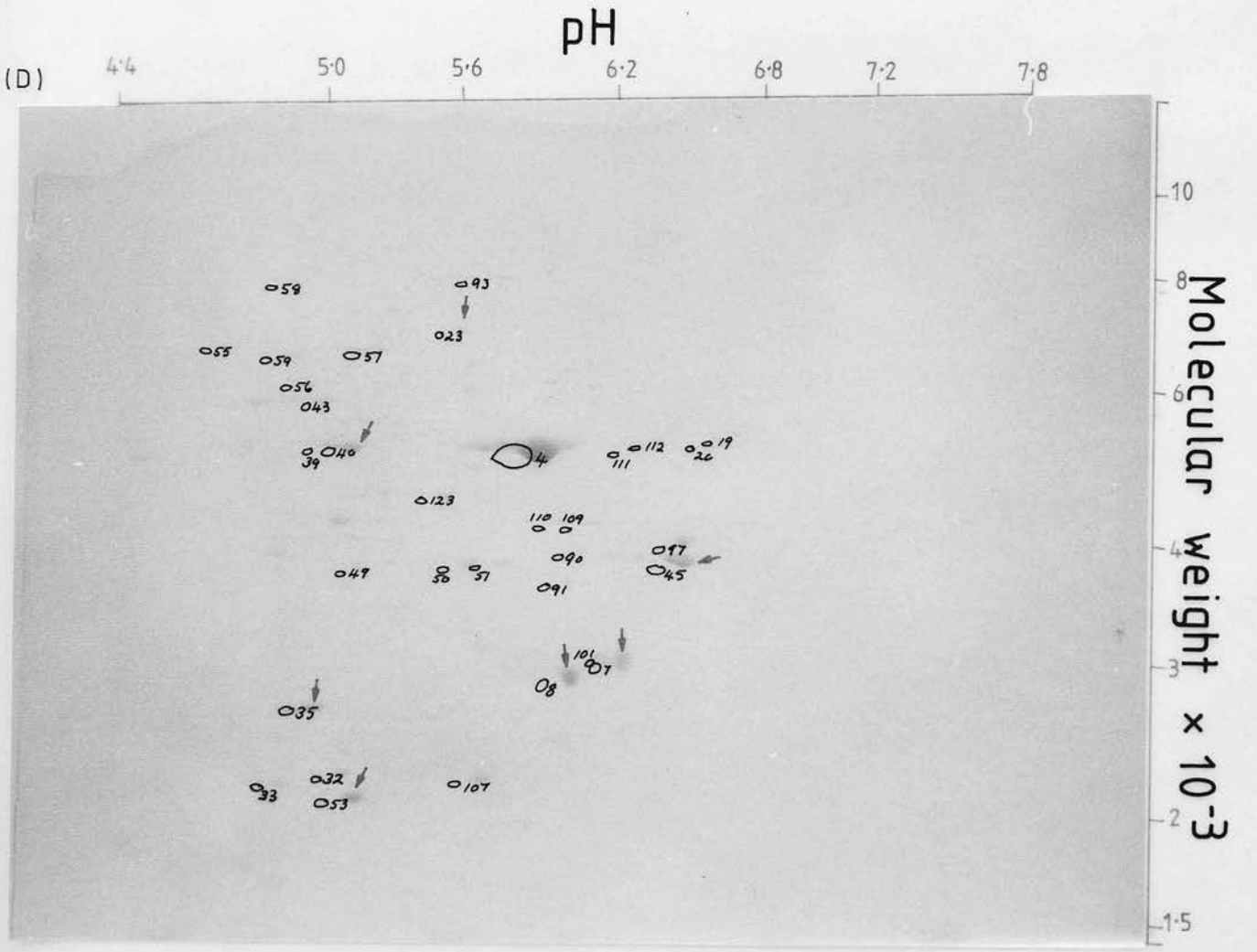
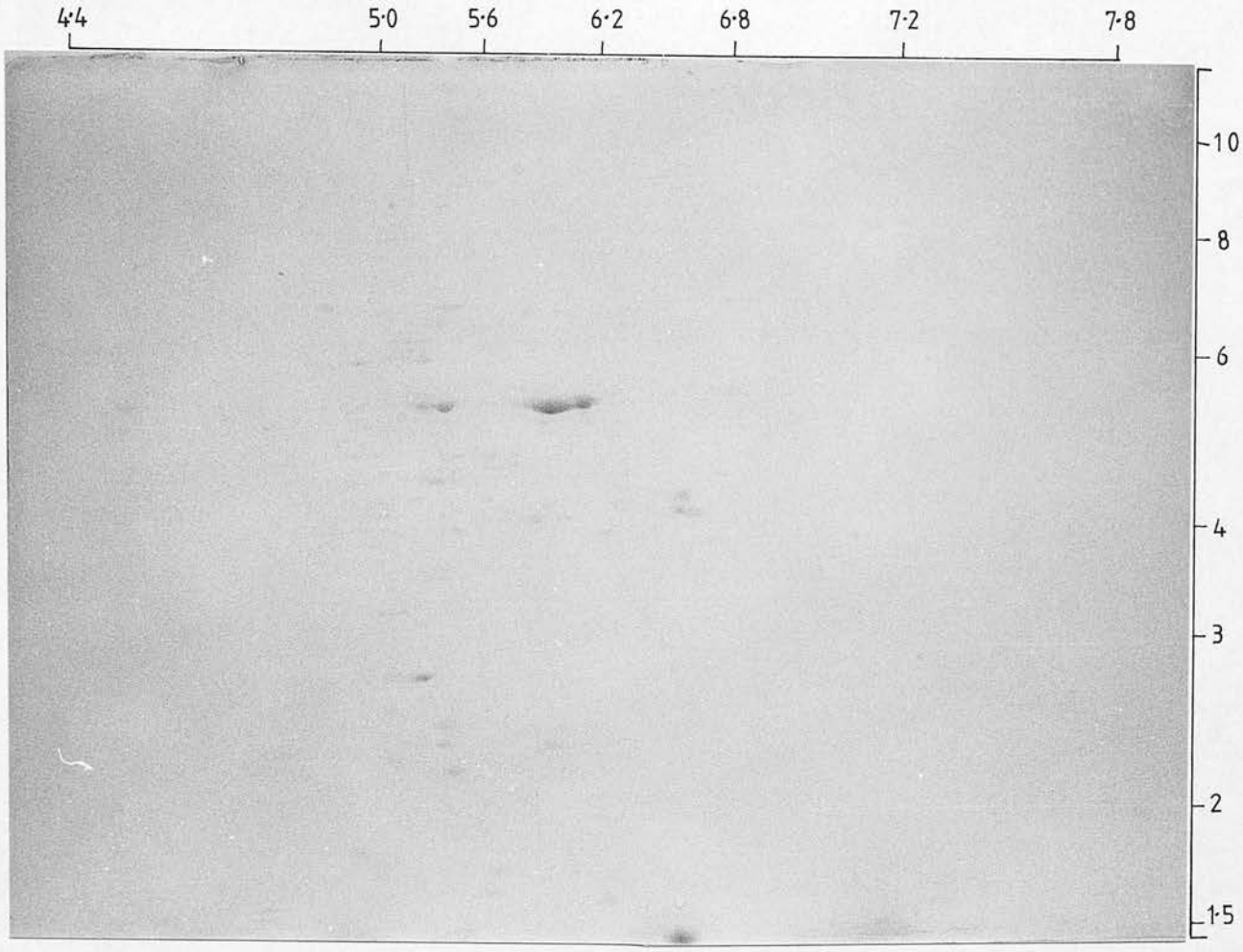

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

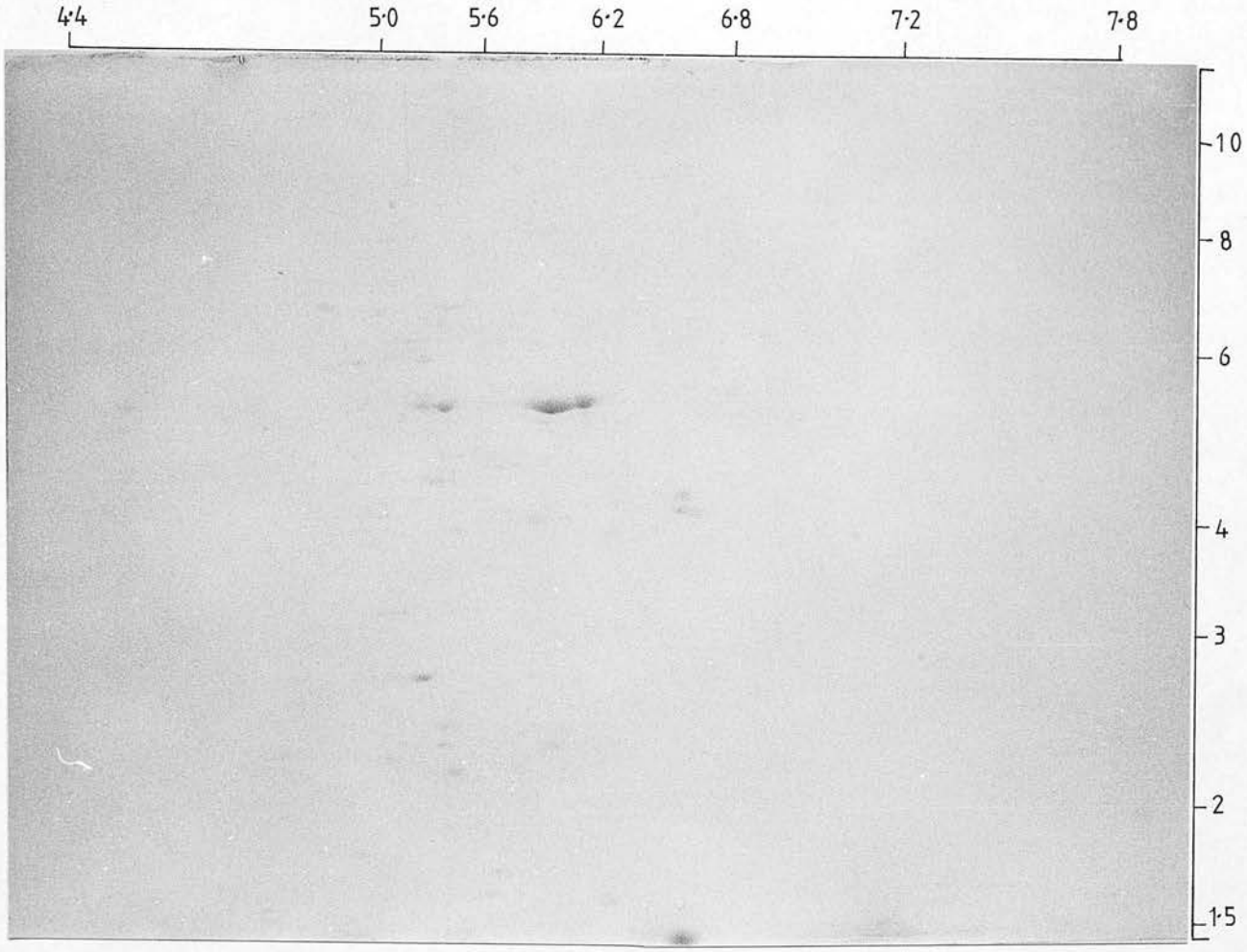
FIGURE 39


(C)
4·4
5·0
5·6
6·2
pH
6·8
7·2
7·8
10
8
6
4
3
2
1·5
Molecular weight × 10-3
059
057
043
041
040
4
045
035
119
032
033
053
076
077
068
069


(D)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5
Molecular weight × 10-3
058
093
023
055
059
057
056
043
039
040
4
111
112
020
019
123
110
109
090
097
049
050
057
045
091
101
107
08
035
032
033
053
107


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

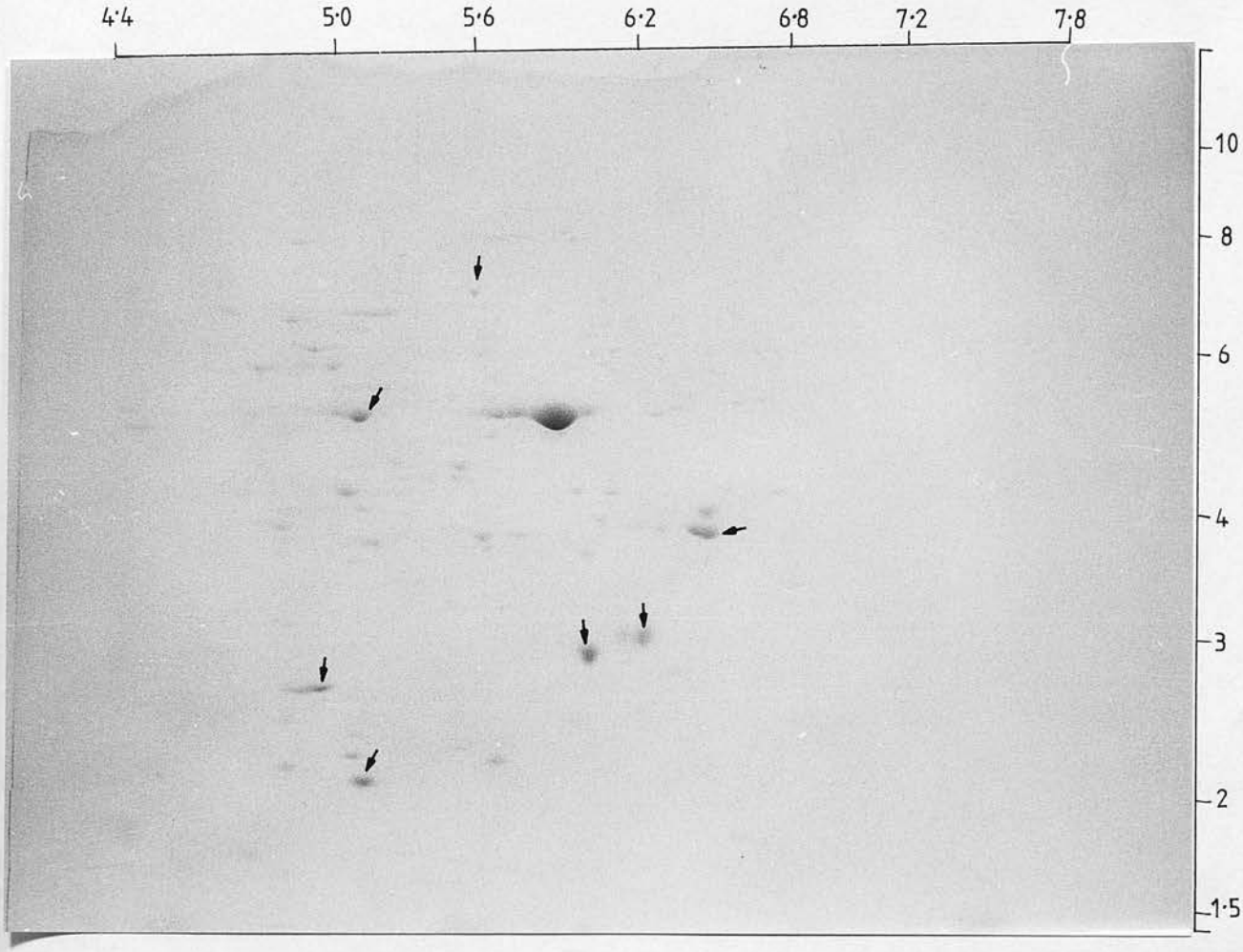
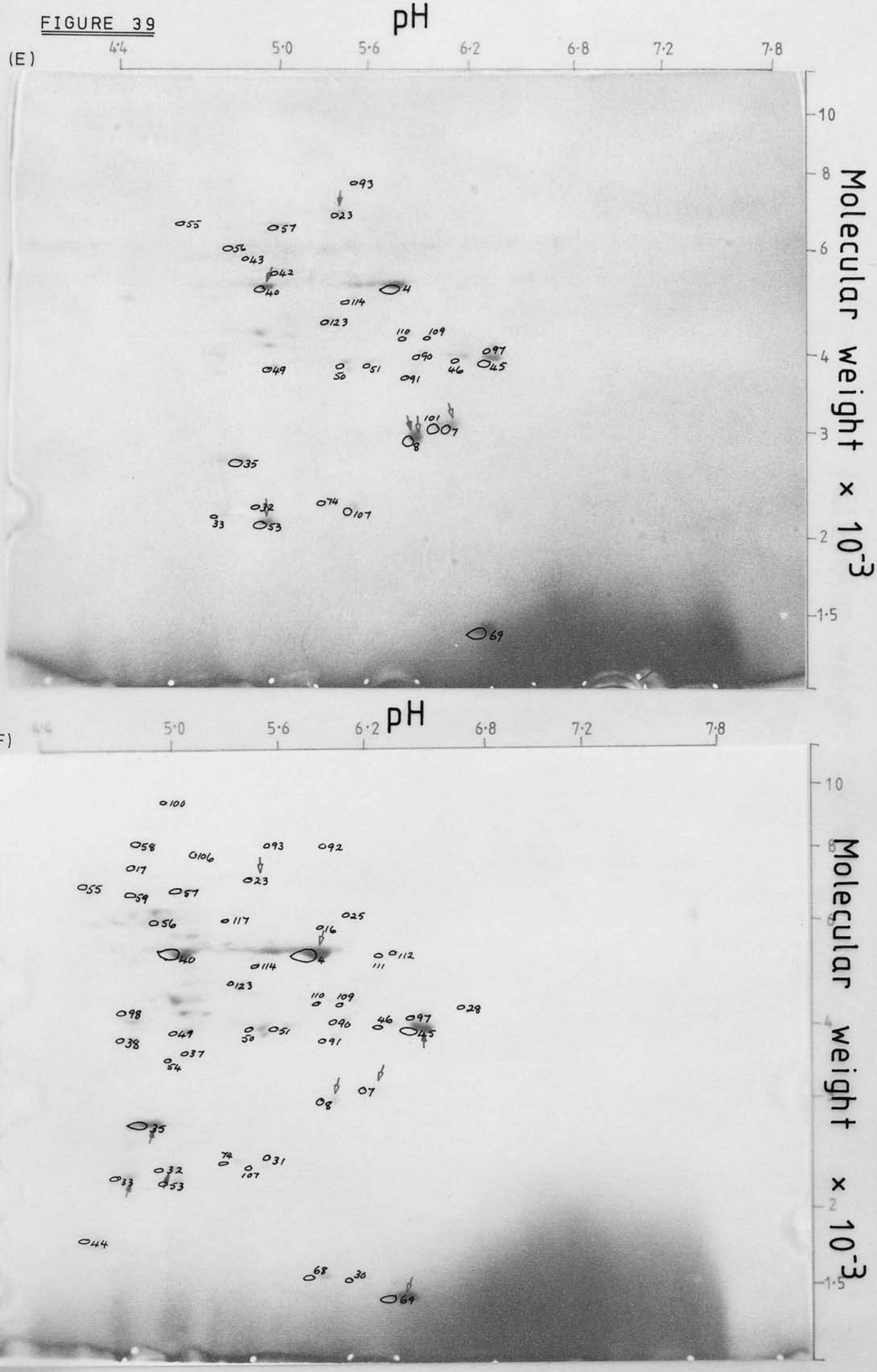
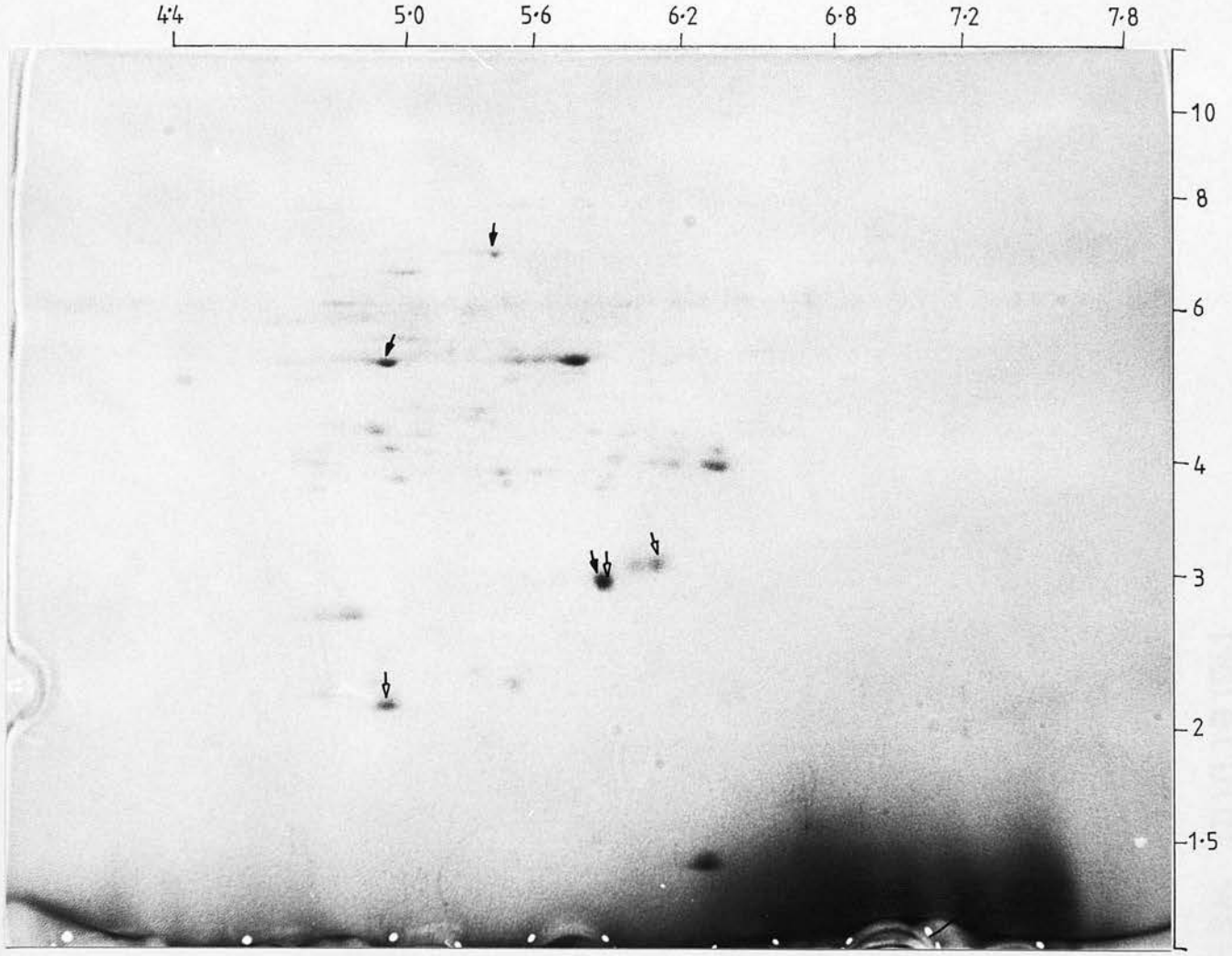

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 39


(E)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
093
023
055
057
056
043
042
040
004
114
123
110
109
097
090
046
045
049
050
051
091
101
007
008
035
074
107
032
033
053
069
(F)
pH
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
4
2
1·5
100
058
106
093
092
017
023
055
059
057
025
056
117
016
040
004
112
111
114
123
110
109
028
098
090
046
097
049
050
051
091
045
038
037
054
007
008
035
074
031
032
107
033
053
044
068
030
069


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

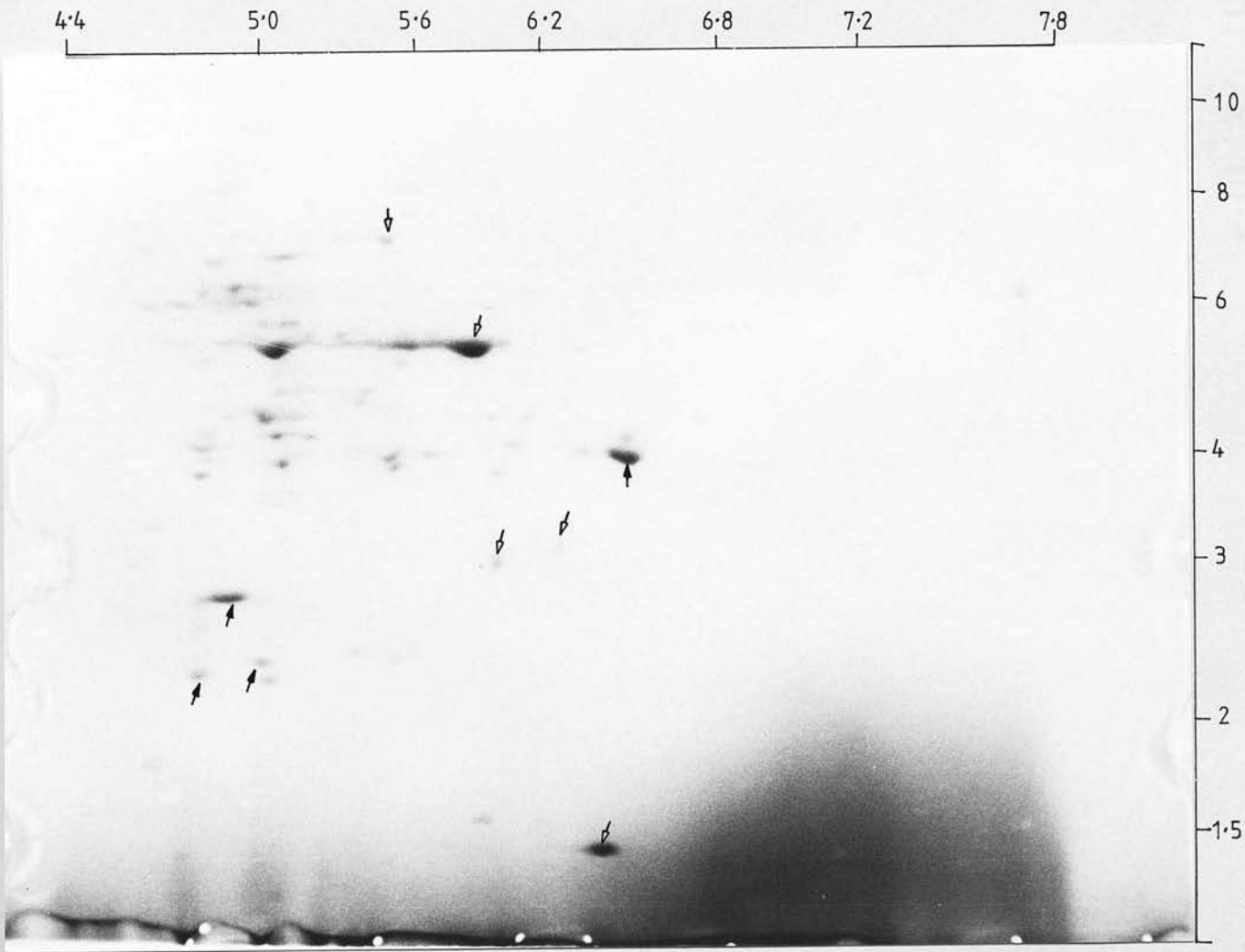
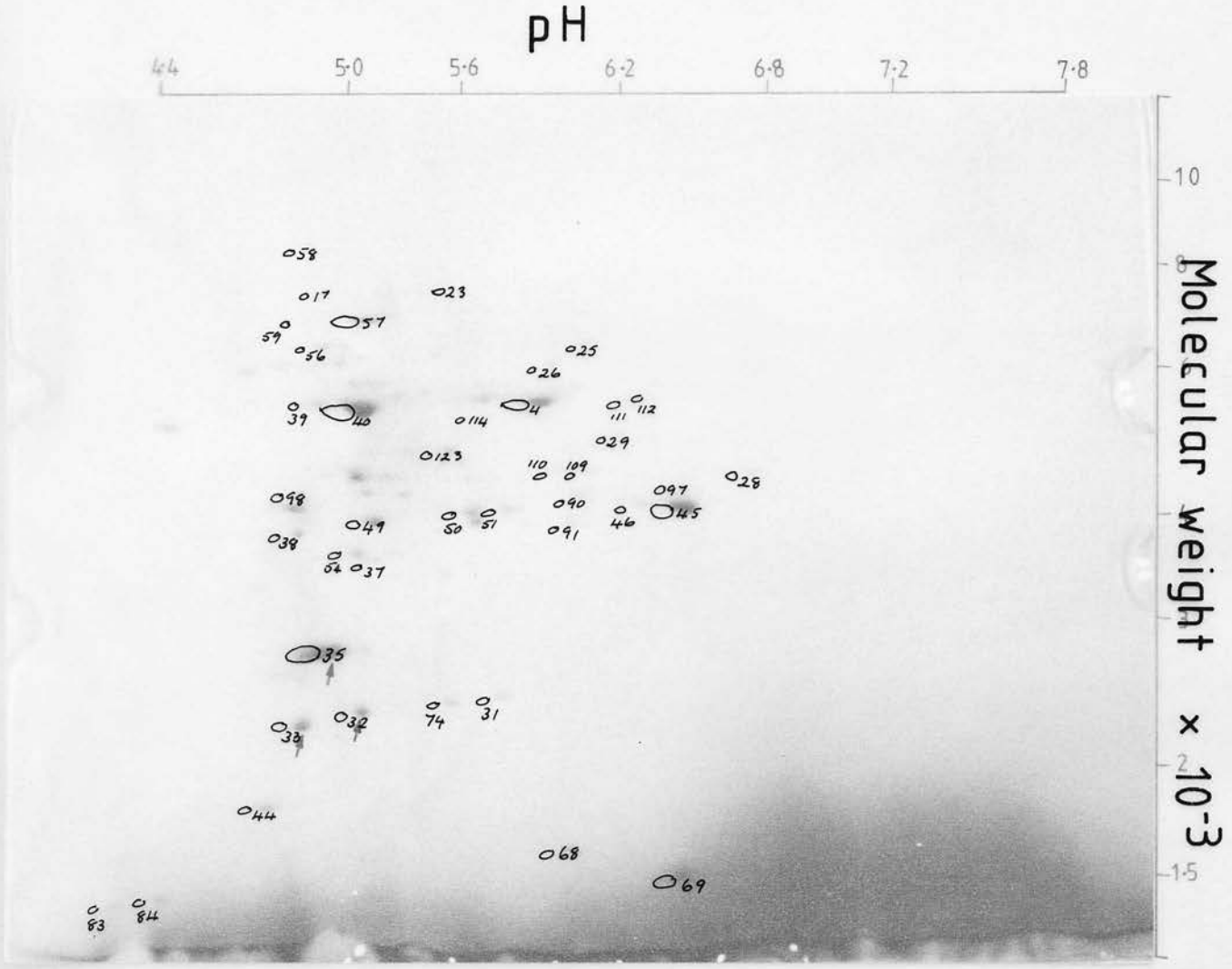
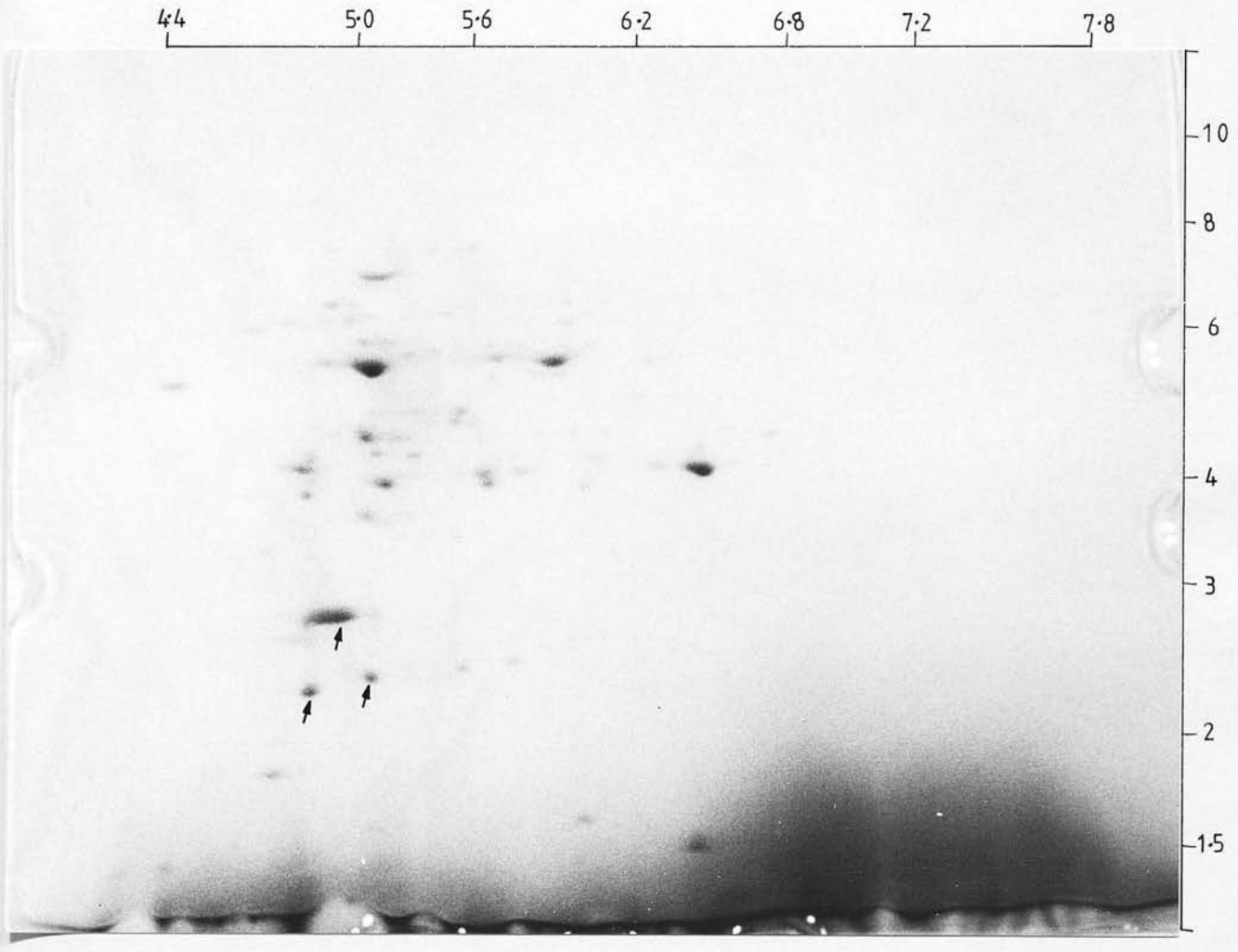

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 39

(G)




4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

amount after 3 days in ABA and proteins 32, 33, 49, 50, 51, 54 and 57 which increase after 5 days. Table 6 shows a list of the most prominent proteins and indicates their position on the gels and their relative abundance throughout turion formation.

The effect of ABA on the mother frond protein was less dramatic but some species were affected (Fig. 40). The major change found in the developing turions i.e. decrease in the relative abundance of protein 4 and increase in protein 40, was not seen in the mother fronds. Proteins 7 and 8, which appear as transient proteins during turion formation, increased in amount during incubation in ABA in the mother fronds, although they reached their maximum concentration 3 days after ABA addition. These proteins increased after 4 days in the developing turion but disappeared after 7 days. In the mother fronds however, the levels found at day 7 were still higher than the levels found in untreated fronds. Protein 45 which increased during turion formation also increased in the mother fronds after only 2 days treatment with ABA. Proteins not changed in relative abundance during the development of the turion but only in the mother fronds were proteins 13, 74 and 124. Proteins 13 and 74 increased in abundance in the mother fronds during incubation in ABA.

The proteins discussed above are those which showed a continuing trend (either increasing or decreasing) during incubation in ABA. Many other proteins showed fluctuations during ABA incubation and these are shown on figures 39 and 40 indicated by arrows.

It should be stressed that equal amounts of soluble protein were applied to the gels within each figure, and a decline or increase in a particular proteins does not indicate an increase or

Table 6: Catalogue of stained soluble proteins during turion formation in S. polyrrhiza (N)

Protein	Molecular weight (daltons)	pI	Occurrence
4	53,500	5•88	decreases throughout turion formation
23	70,000	5•55	increases day 3 & thereafter
28	44,000	6•80	appears day 5 and remains
32	23,000	5•06	increases day 5 & thereafter
33	22,000	4•95	increases day 5 & thereafter
35	27,000	5•98	increases throughout turion formation
40	52,000	5•06	increases throughout turion formation
45	40,000	6•51	increases day 3 & thereafter
46	40,000	6•42	appears day 3, increasing thereafter
49	37,500	5•12	increases day 5
50	38,500	5•58	increases day 3 & thereafter
51	39,000	5•82	increases day 3 & thereafter
53	21,500	5•07	increases day 3, decreases day 5, disappears day 7
54	34,000	5•06	increases day 5 & thereafter
55	67,000	4•92	disappears day 7
57	67,000	5•06	increases day 5 & thereafter
59	73,000	4•91	increases day 7
97	42,000	6•51	decreases day 7

Figure 40

Pattern of Coomassie blue stained proteins during incubation of the mother fronds associated with the turions in 1×10^{-7} M ABA. Soluble protein was extracted from each day after ABA addition and loaded onto 2 - dimensional gels. Equal amounts of soluble protein were applied to each gel (100 µg).

a) day 0 (untreated)

b) day 1

c) day 2

d) day 3

e) day 4

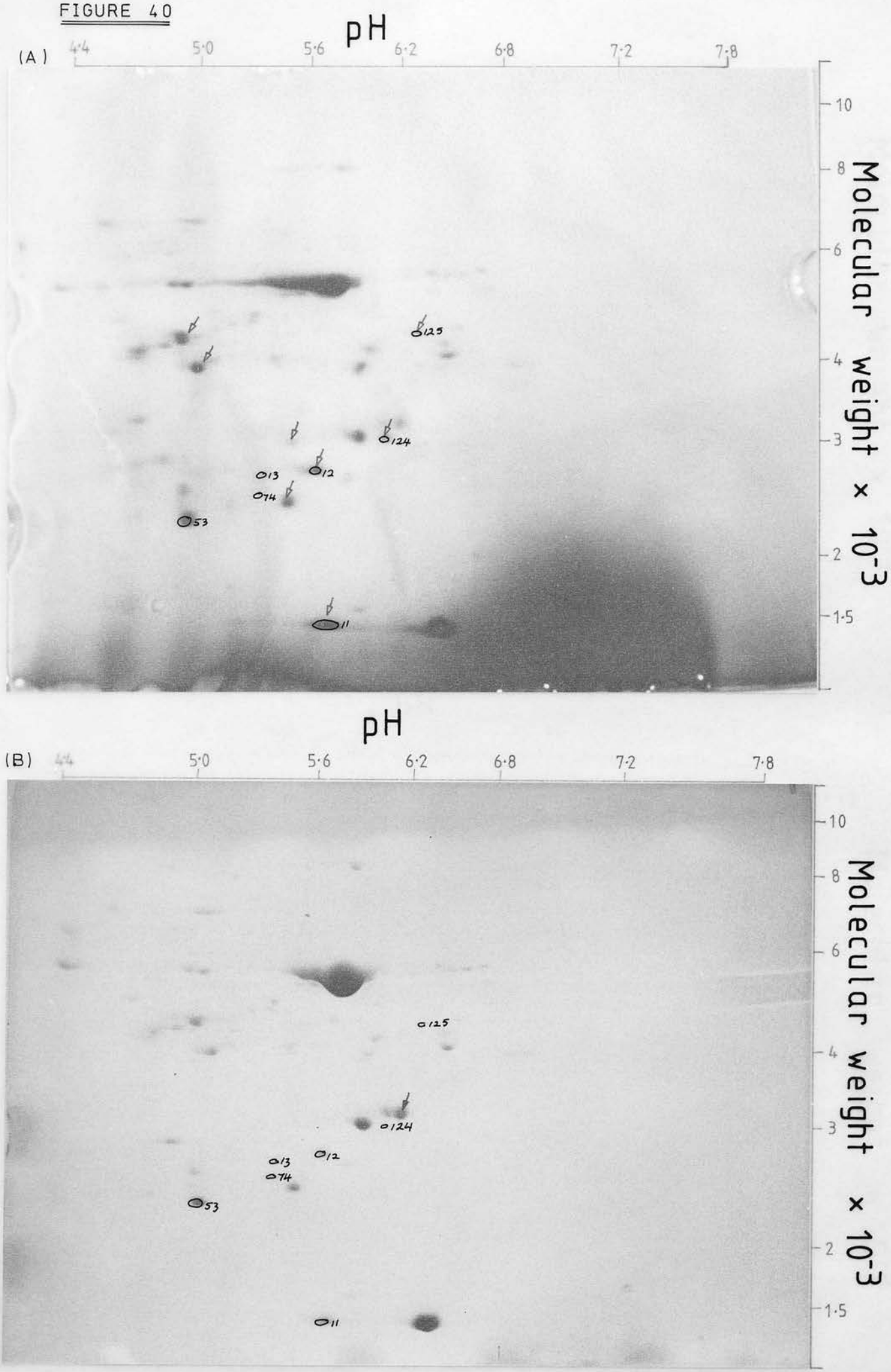
f) day 5

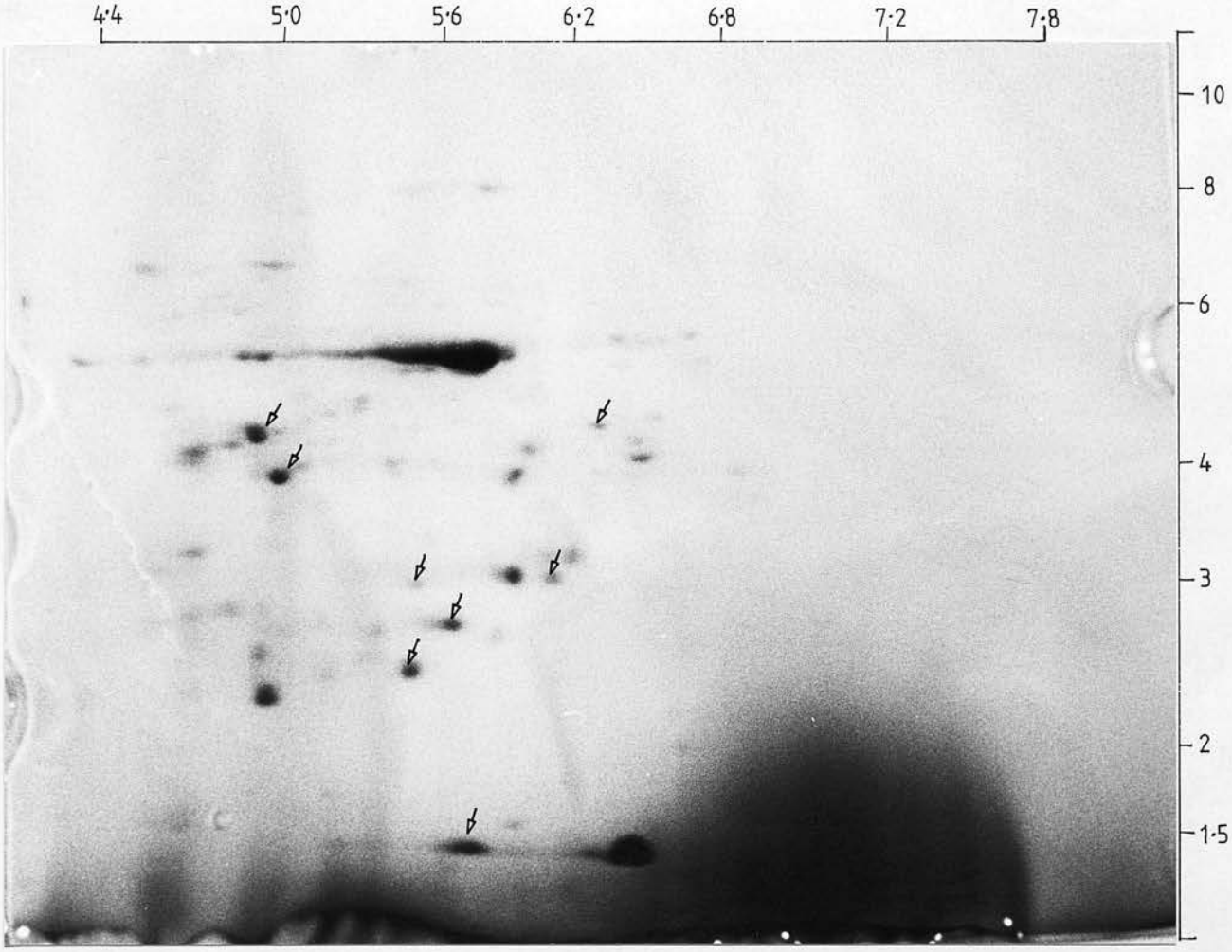
g) day 7

↘ indicates that the protein has increased in amount from the day before

↘ indicates that the protein has decreased in amount on the next day

FIGURE 40


(A)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10⁻³
10
8
6
4
3
2
1·5
O125
O124
O13
O12
O74
O53
O11
(B)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10⁻³
10
8
6
4
3
2
1·5
O125
O124
O13
O12
O74
O53
O11


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

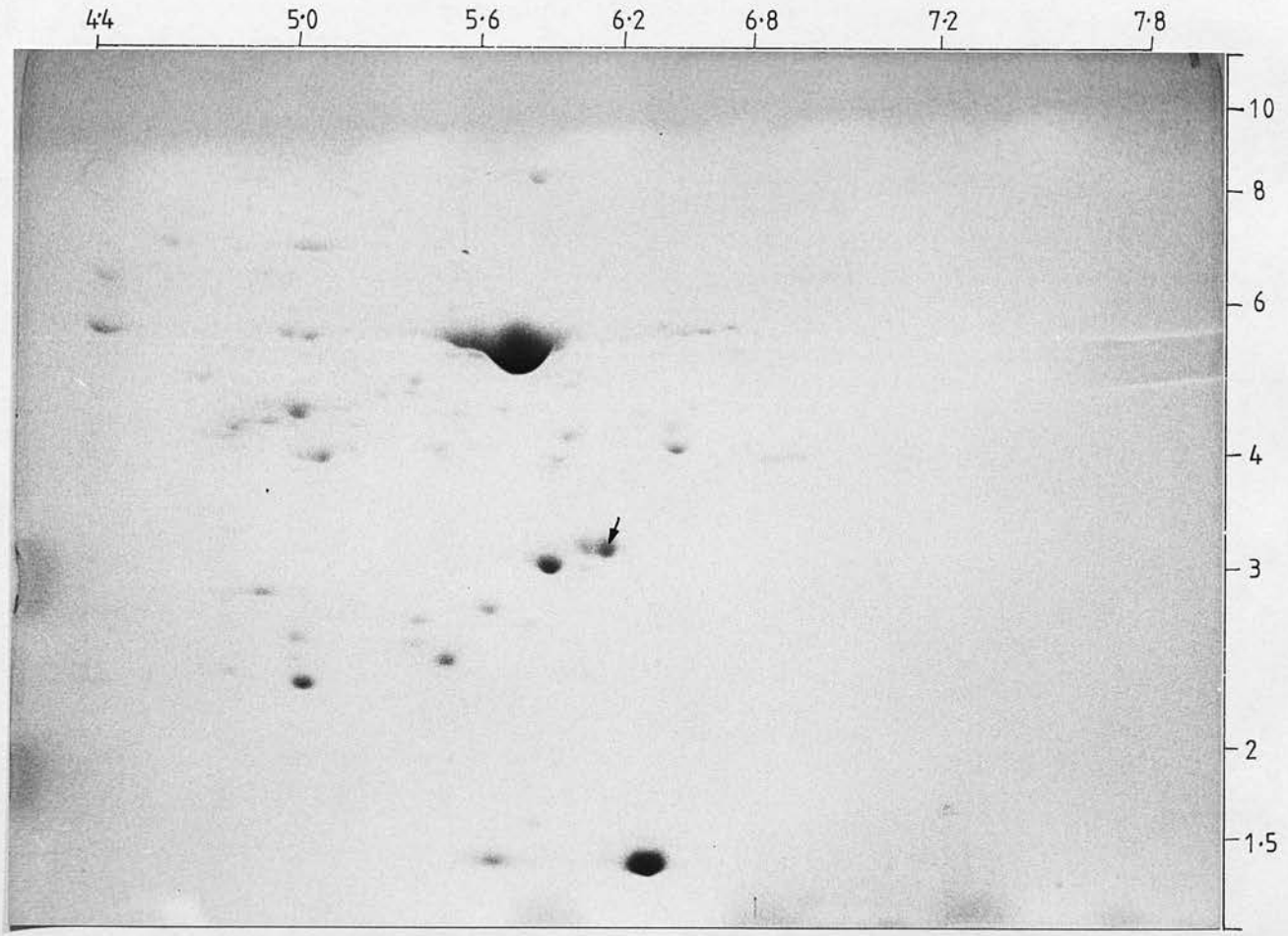
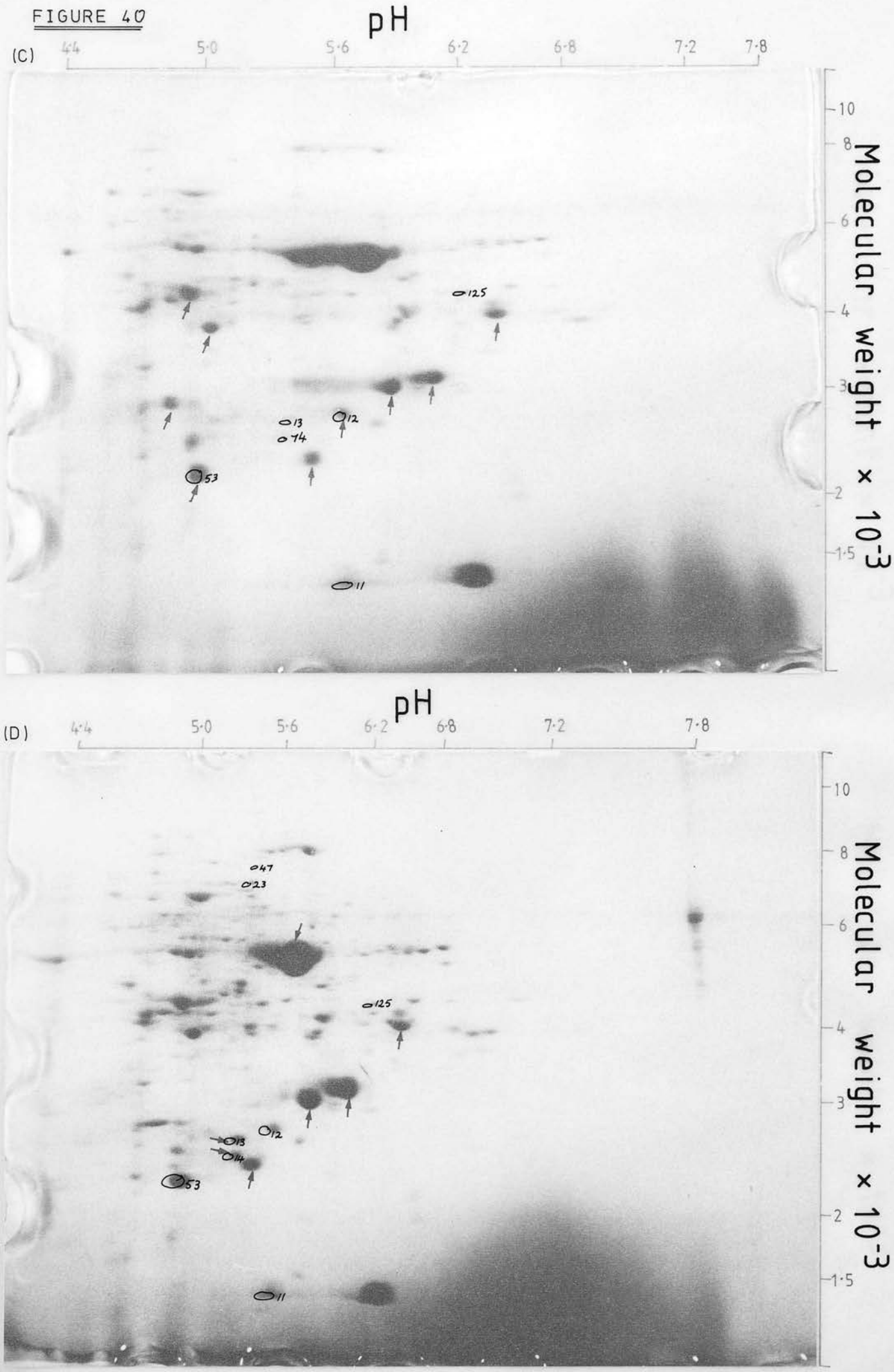
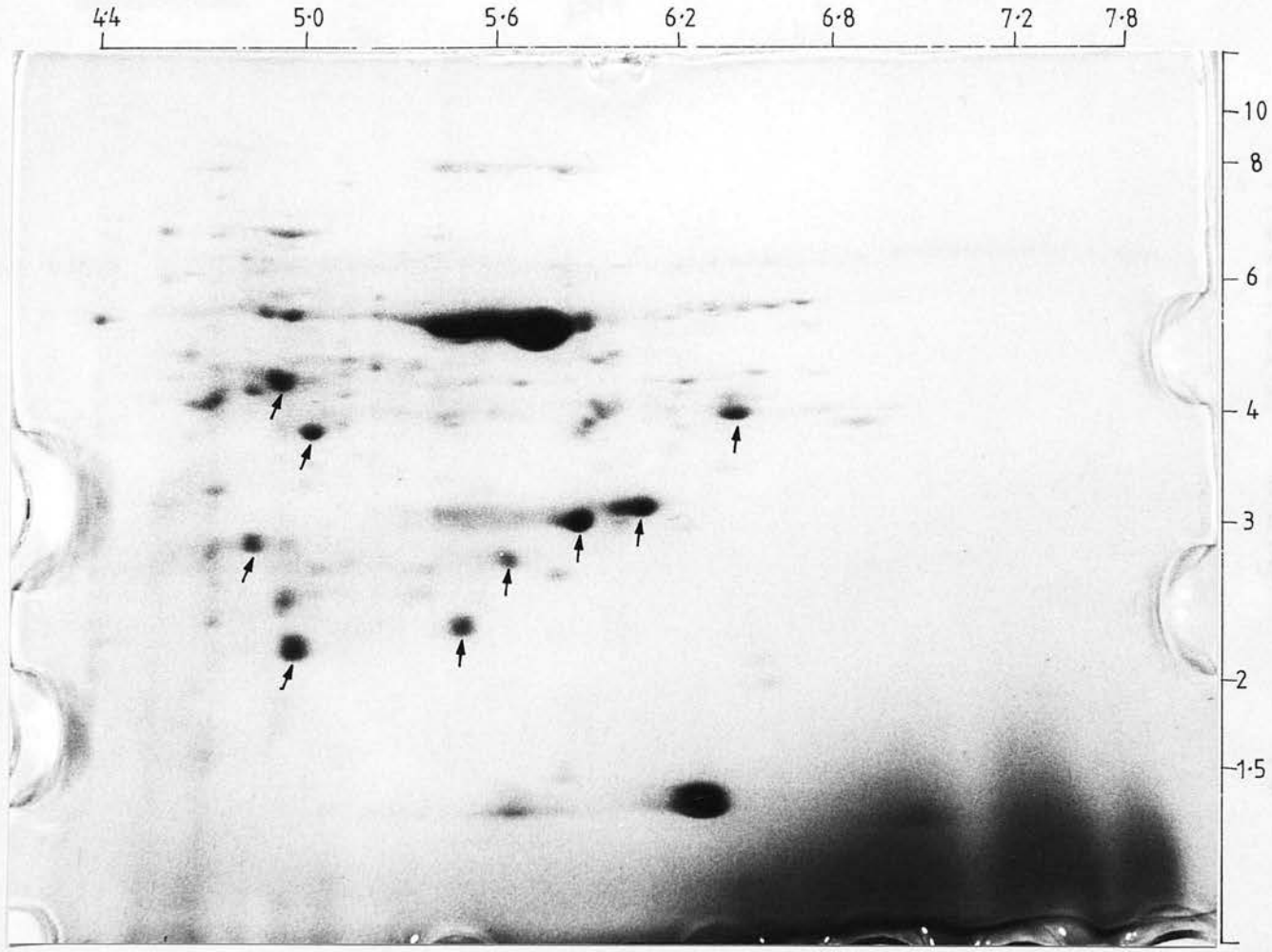

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 40


pH
(C)
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
125
13
12
74
53
11
pH
(D)
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
47
23
125
12
13
14
53
11


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

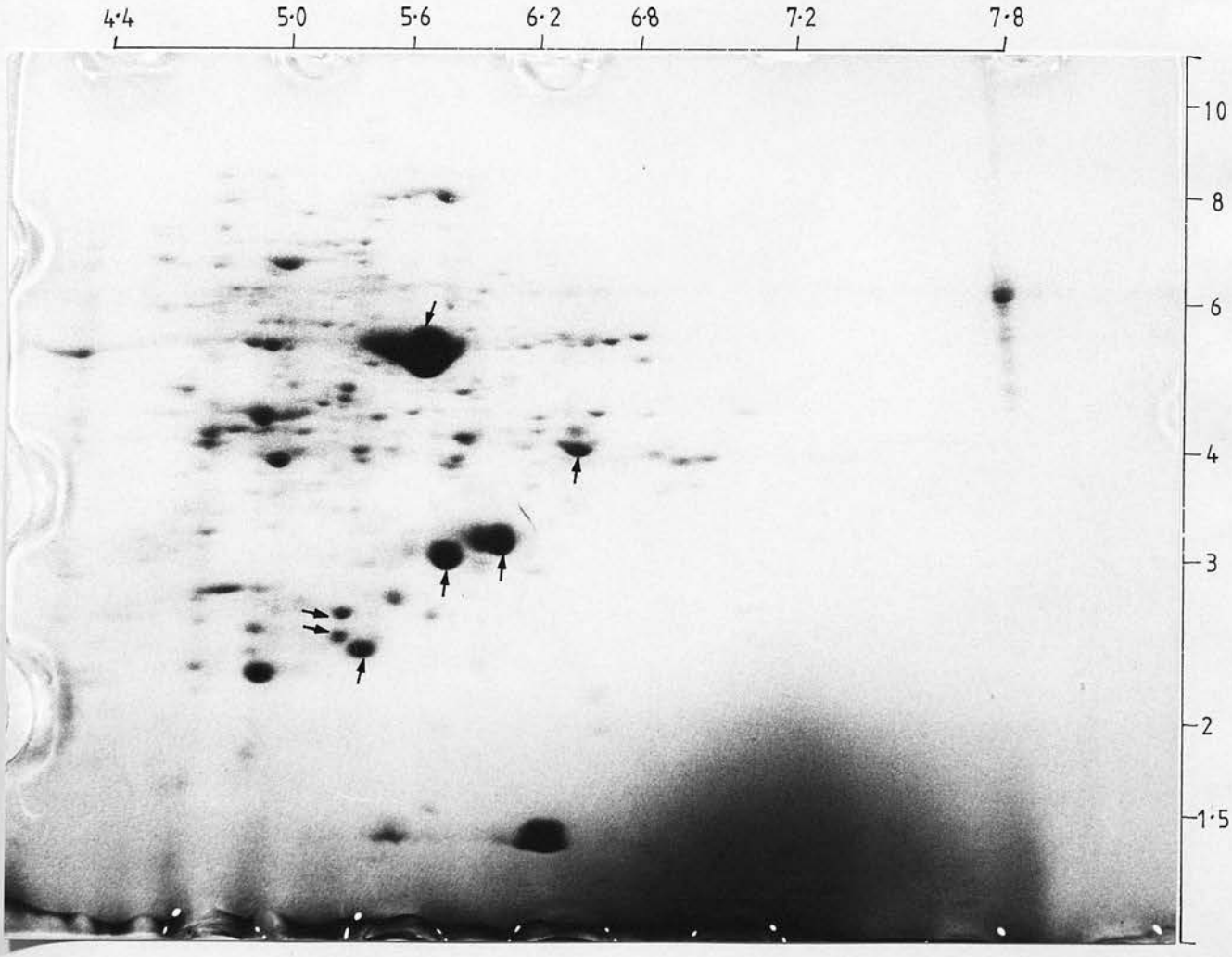
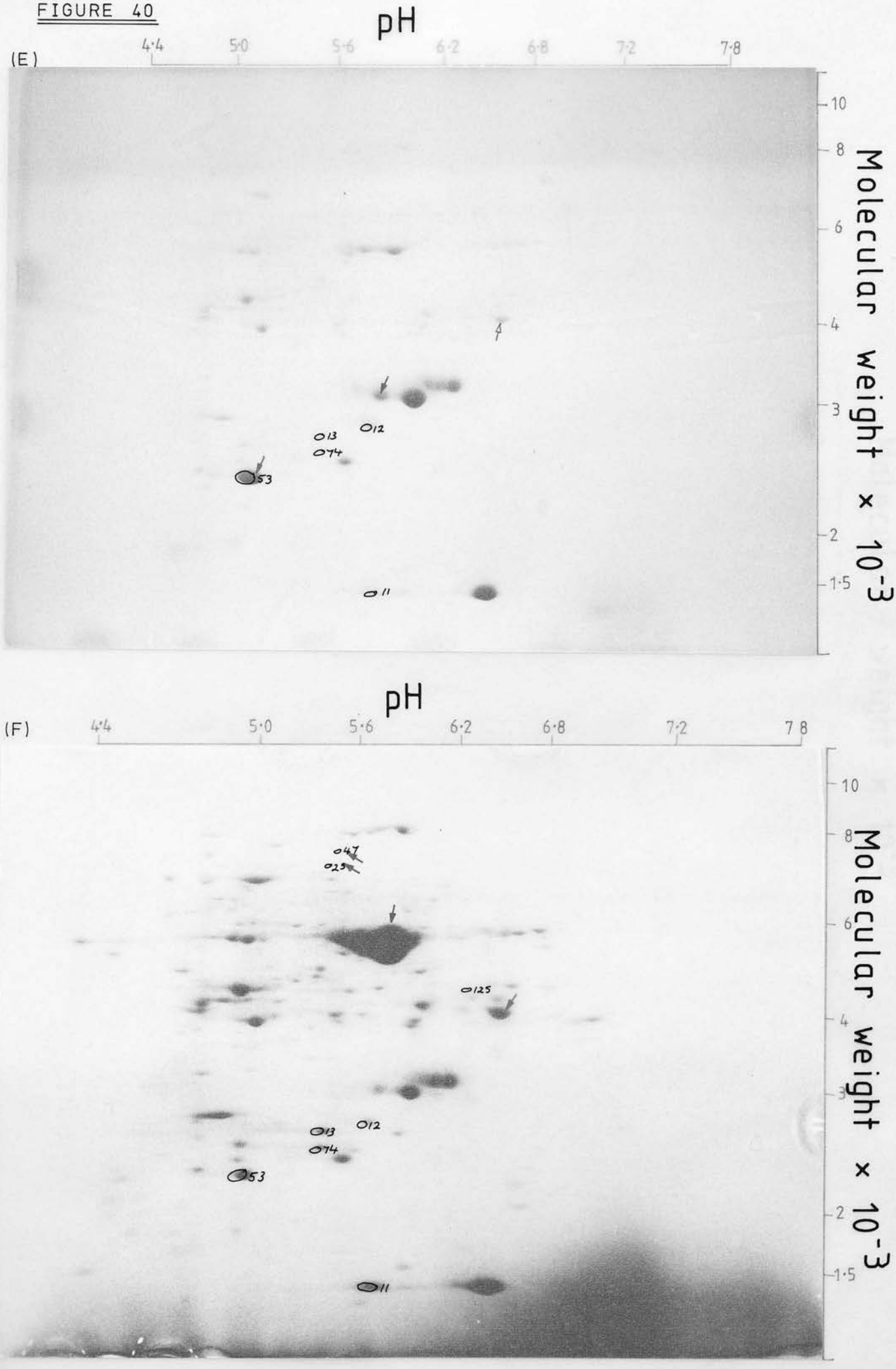
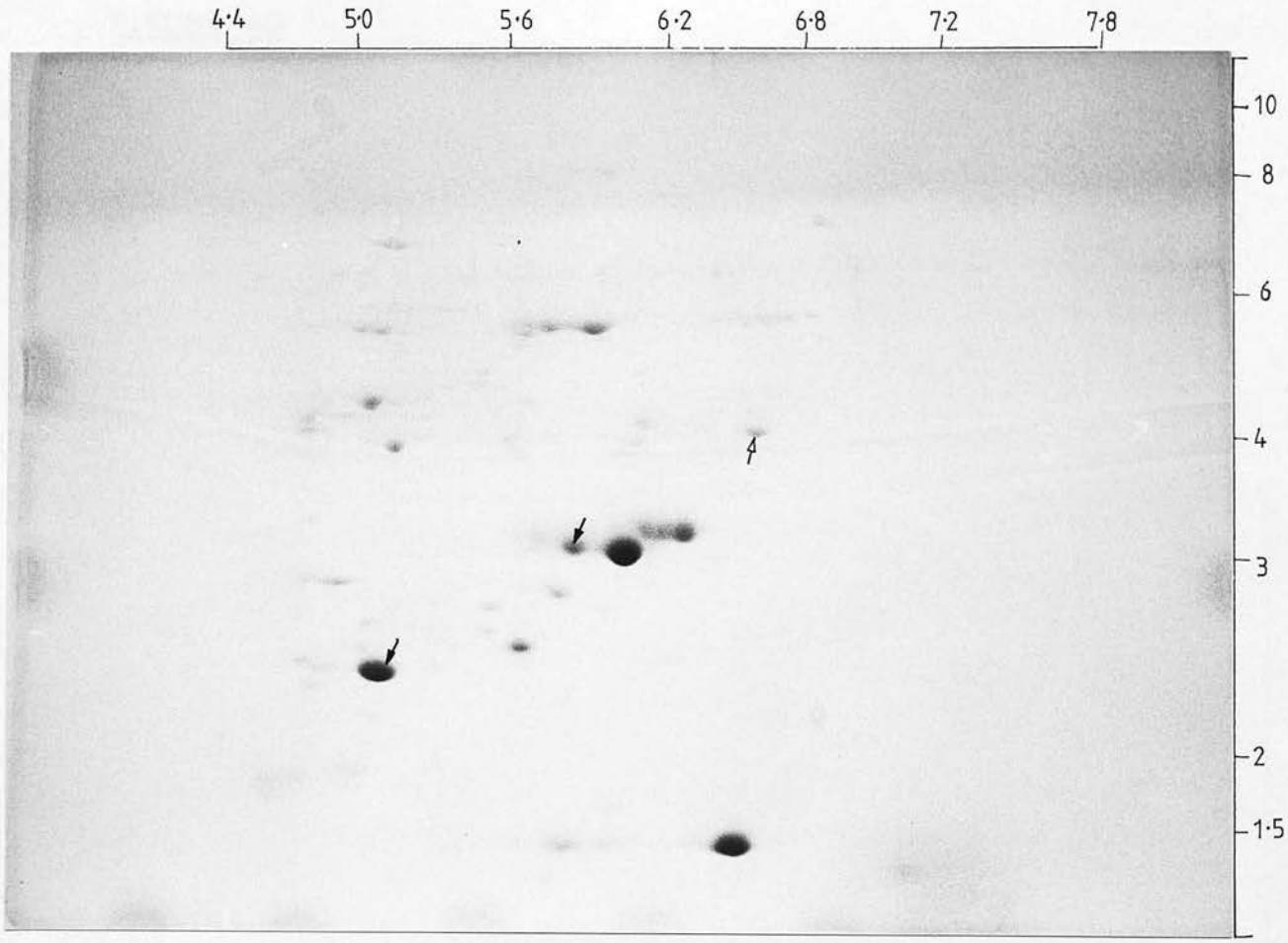

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

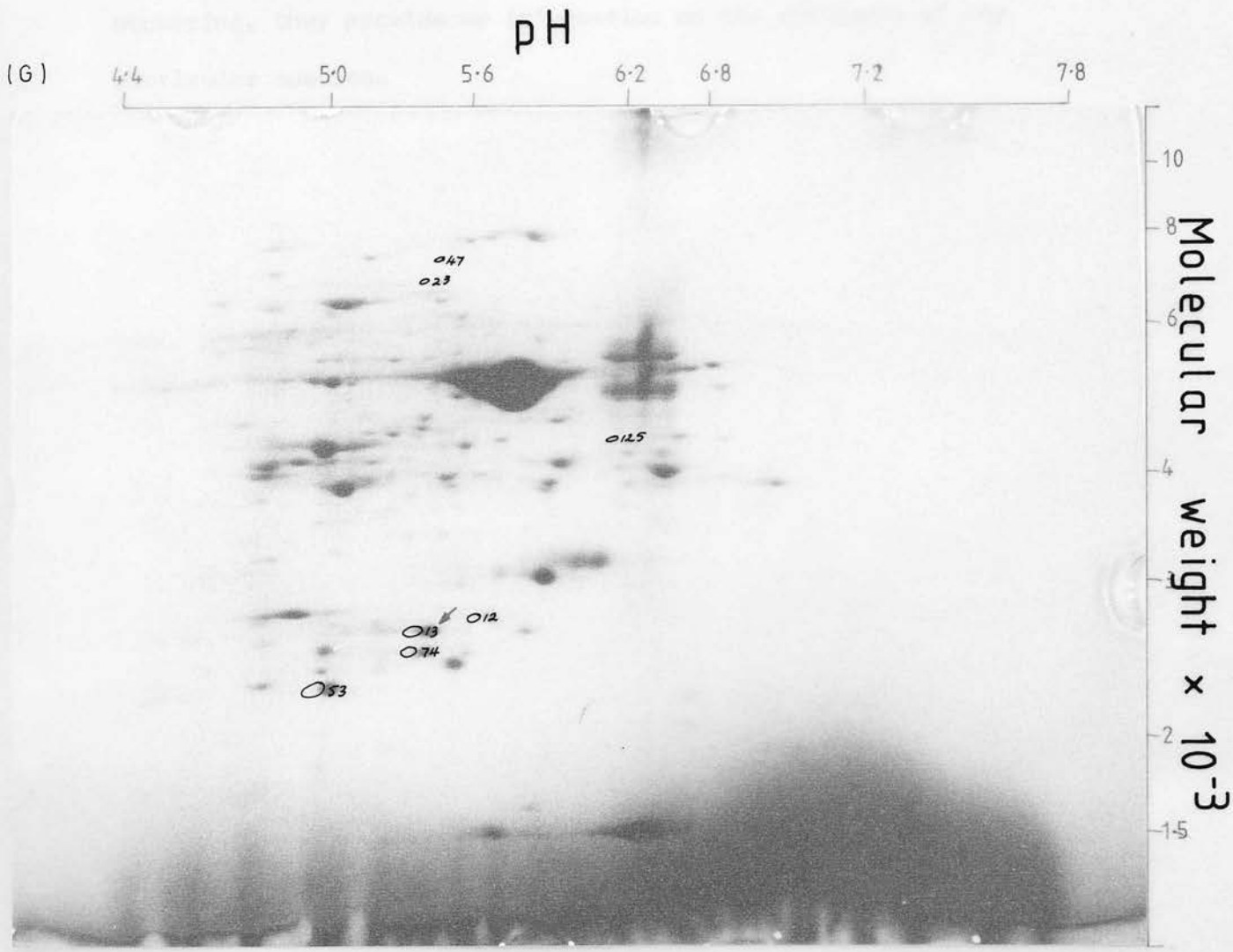
FIGURE 40

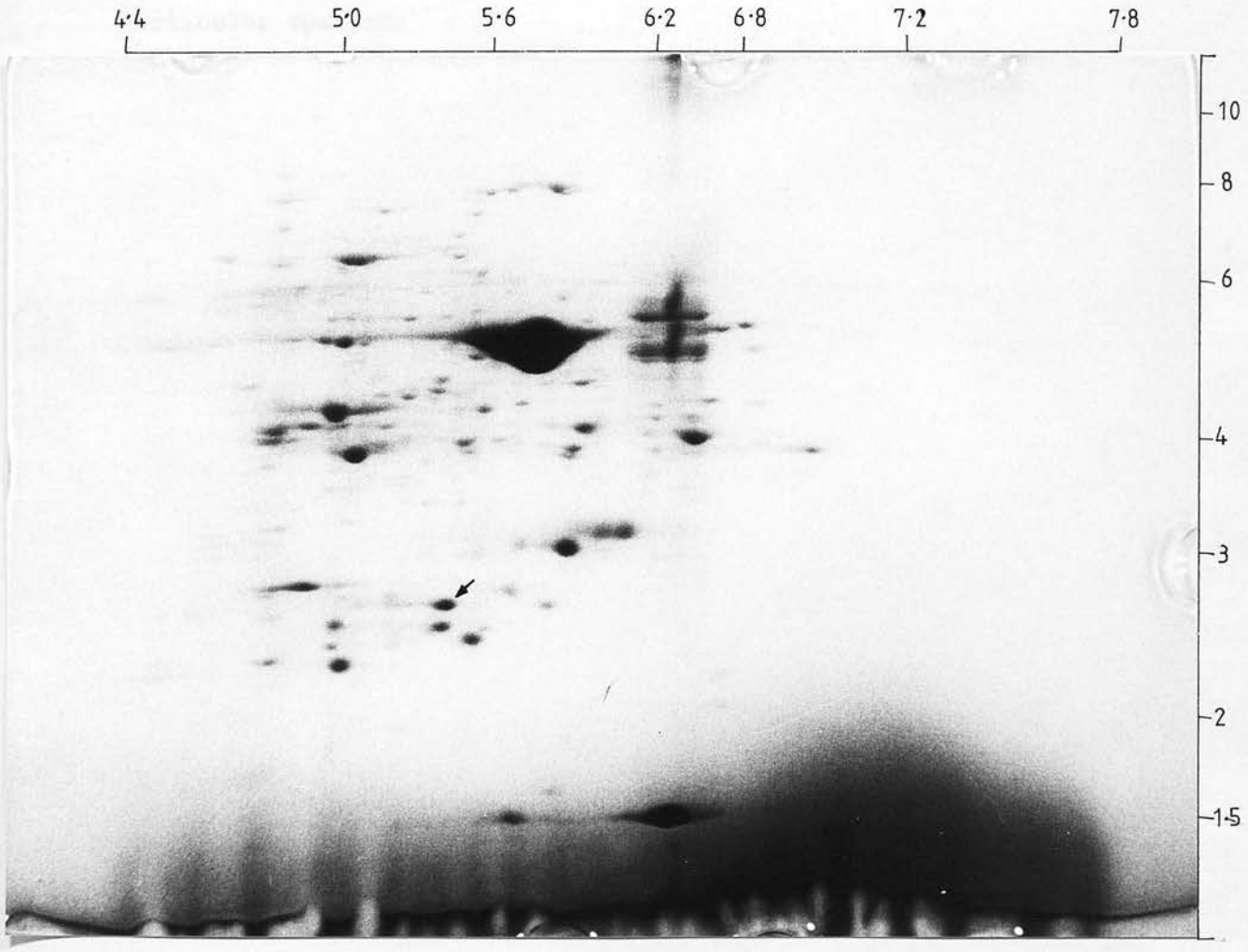

(E)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
013
012
074
053
011
(F)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
047
023
0125
013
012
074
053
011


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5


4·4
5·0
5·6
6·2
6·8
7·2
7 8
10
8
6
4
3
2
1·5

FIGURE 40


(G)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10⁻³
10
8
6
4
2
1·5
047
023
0125
012
013
074
053


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

decrease in the amount of that protein, merely an altered abundance relative to the bulk of the soluble protein present at each developmental stage. Although stained gels of the protein patterns during turion formation indicate that there are specific changes occurring, they provide no information on the synthesis of any particular species.

4.6 DNA LEVELS DURING TURION FORMATION

As the vegetative frond of S. polyrrhiza develops it increases its DNA content dramatically, only declining when the frond approaches senescence. Ultrastructural analysis indicated that the main period of cell division is probably completed by the time a frond is 2 mm long, and indeed the rate of the increase in the DNA content of the frond declines above this size (Fig. 41). However the frond continues to accumulate DNA until maturity, although it seems unlikely that the increased DNA content results from increased cell numbers within the frond itself. Although the possibility of polyploidy, gene amplification, or stimulation of chloroplast replication cannot be ruled out during the normal development of the vegetative frond, the increased DNA content is more likely to be due to the internal structure of the frond itself.

The frond of Spirodela, even at early stages in its development is quite unlike the leaves of most angiosperms in that it contains 2 meristematic pockets at its proximal end. During dissection of a culture into the various developmental stages, it is not possible to remove all these areas from the fronds. For example, a frond of 2 mm would probably have up to 4 generations of primordia within its

Figure 41

DNA levels during turion development and in the associated mother fronds incubated in 1×10^{-7} M ABA; and DNA levels during the development of the vegetative frond of S. polyrrhiza (N). DNA was extracted by the method of Guinn and determined by the diphenylamine assay.

● developing turions

○ mother fronds

developing vegetative fronds

Levels on a gram fresh weight basis:

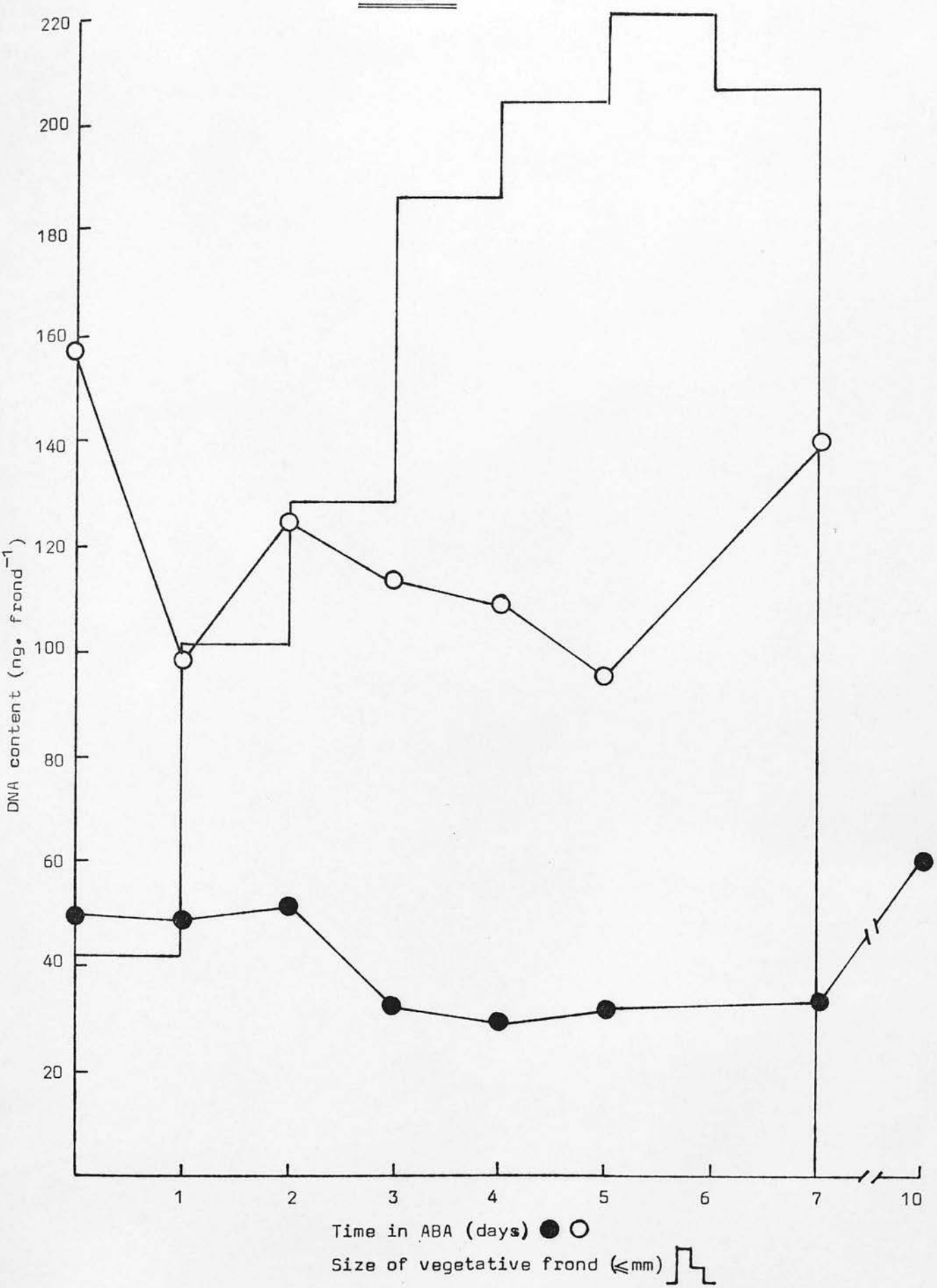
Developing turions = 267 → 94 $\mu g. g^{-1}$ (115 $\mu g. g^{-1}$)*

Mother fronds = 48·5 → 37·1 $\mu g. g^{-1}$

Developing vegetative fronds = 227 → 52 $\mu g. g^{-1}$

* fully mature turions at day 10

FIGURE 41


220
200
180
160
140
120
100
80
60
40
20
DNA content (ng. frond^{-1})
1
2
3
4
5
6
7
10
Time in ABA (days) ● ○
Size of vegetative frond (≤mm)

meristematic pockets. The cells of these tiny primordia would be rapidly dividing and would contain large amounts of DNA.

The developing turion however, shows no such increase in its DNA content, and when expressed on a fresh weight basis there is a decline from approximately 247 μg. g^{-1} to 115 μg. g^{-1} in the fully developed turion after 10 days in ABA. The increase in DNA content shown by the developing vegetative frond would not be expected in the developing turion, since the latter has only quiescent primordia within its meristematic pockets. ABA had no detectable effect on the DNA content of the associated mother fronds. Even on a per frond basis the developing turion showed a slightly decreased DNA content during its development in ABA which could possibly indicate that DNA synthesis is lowered during turion formation and/or degradation is enhanced.

4.7 RNA LEVELS DURING TURION FORMATION

The RNA content of the vegetative frond increases with its size just as DNA levels do, but with no decline at full maturity (Fig. 42). No such increase is found in the developing turion during its development in ABA, and the RNA content per frond declines slightly. On an approximate fresh weight basis, the developing frond has less RNA at maturity (1·03 mg. g^{-1}) than at the ≤ 1 mm size (2·70 mg. g^{-1}) and in the mature turion the level has declined to only 1·40 mg. g^{-1} RNA. In the developing turion there generally appears to be a less pronounced effect on RNA levels than on DNA levels, possibly indicating that synthesis of the major RNA species is affected to a lesser extent than DNA synthesis during turion formation (see Table 7).

Figure 42

RNA levels during turion development and in the associated mother fronds incubated in 1×10^{-7} M ABA; and RNA levels during the development of the vegetative frond of S. polyrrhiza (N). RNA was extracted by the method of Guinn and determined spectrophotometrically.

● developing turions

○ mother fronds

developing vegetative fronds

Levels on a gram fresh weight basis:

Developing turions = 2·9 → 1·2 mg. g^{-1} (1·4 mg. g^{-1})*

Mother fronds = 1·1 → 1·2 mg. g^{-1}

Developing vegetative fronds = 2·5 → 1·0 mg. g^{-1}

* fully mature turions at day 10

FIGURE 42

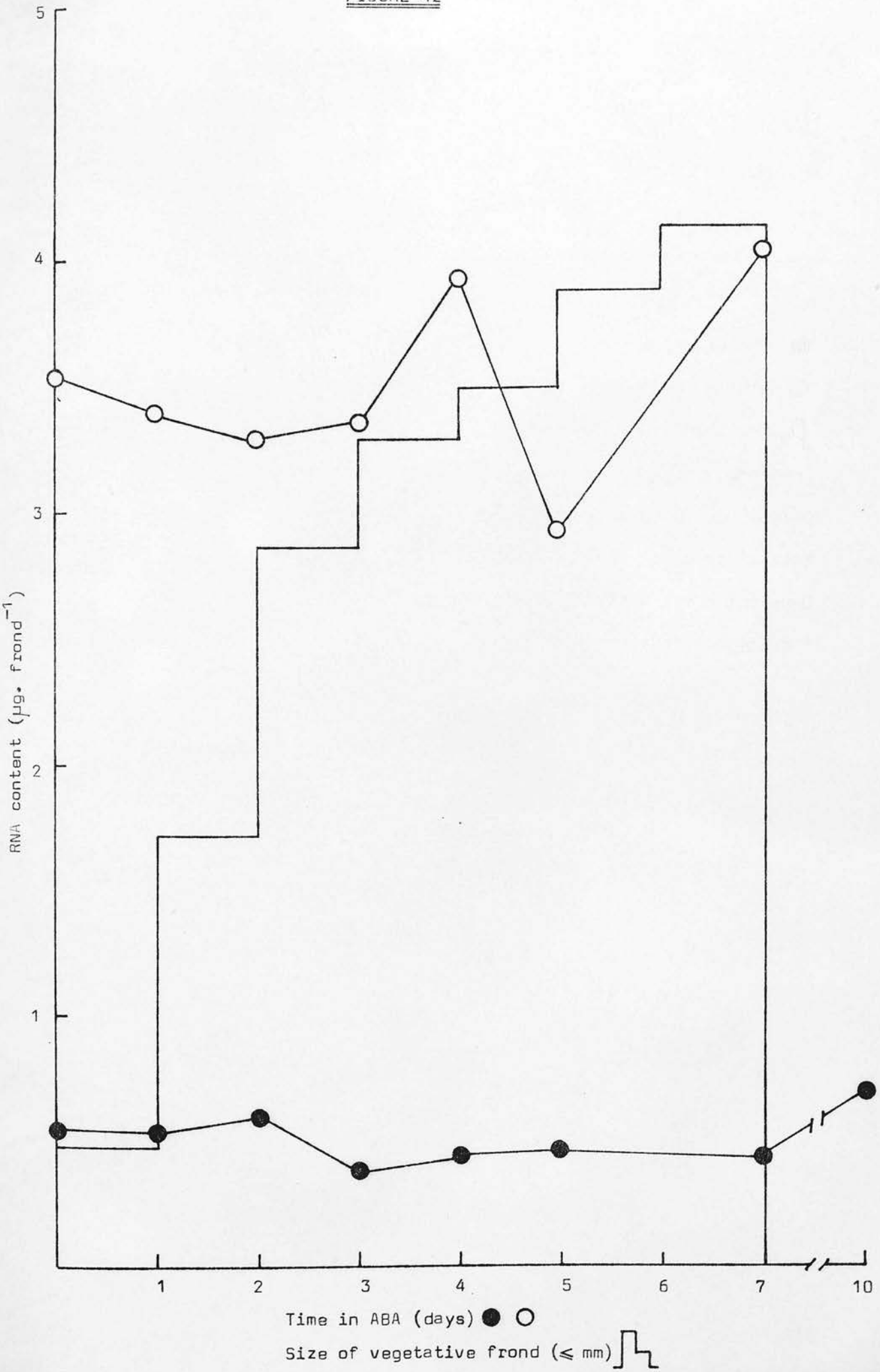

5
4
3
2
1
RNA content (µg. frond^{-1})
1
2
3
4
5
6
7
10
Time in ABA (days) ● ○
Size of vegetative frond (≤ mm)

Table 7: Comparison of protein, RNA and DNA levels between the developing turion and associated mother fronds in 1×10^{-7} M ABA and the developing vegetative frond of S. polyrrhiza (N)

Macromolecule	Developing frond 1 mm – 7 mm	Developing turion day 0 – 7	Mother frond day 0 – 7
DNA (ng. frond^{-1})	+ 352%	– 26%	no change
DNA (μg. g^{-1})	– 79%	– 62%	no change
RNA (μg. frond^{-1})	+ 694%	– 15%	no change
RNA (mg. g^{-1})	– 63%	– 56%	no change
Total protein (μg. frond^{-1})	+ 1195%	+ 98%	+ 48%
Total protein (mg. g^{-1})	– 40%	+ 4%	+ 48%
Soluble protein (μg. frond^{-1})	+ 2746%	– 10%	+ 61%
Soluble protein (mg. g^{-1})	+ 31%	– 53%	+ 61%
Insoluble protein (μg. frond^{-1})	+ 2276%	+ 173%	+ 14%
Insoluble protein (mg. g^{-1})	+ 7%	+ 43%	+ 14%

This table shows the % change in the parameters throughout development or incubation in ABA.

\+ increases by – decreases by

4.8 ANTHOCYANIN LEVELS DURING TURION FORMATION

During the development of the turion, a brown colouration develops first at the distal end and proceeds proximally until the mature turion is completely brown. No increase in pigmentation was seen in the associated mother fronds.

This rise in pigmentation was reflected in the accumulation of anthocyanin during turion formation (Figs. 43 and 44). This accumulation of pigment in S. polyrrhiza (N) was found to be totally associated with the developing turion with a 67 fold increase in the anthocyanin content per frond during its development. There is also an increase in the anthocyanin content of the developing vegetative frond, although the final level in the mature frond actually represents a decrease of 17% of the level found in fronds $\leqslant$ 1 mm in length when expressed on a fresh weight basis (Fig. 43).

The anthocyanin content of the mother fronds associated with the developing turions actually decreased by 45% after incubation in ABA for 7 days. One possible explanation for this is that precursors of anthocyanin synthesis may have been diverted to the developing turion from the mother frond during the process of turion formation, thus resulting in a progressive loss of the pigment from the mother fronds.

The anthocyanins are a class of pigments which may certainly be under the control of phytochrome and/or plant growth regulators, although the response seems to vary with the tissue under study. Anthocyanin levels were increased by cytokinin in Impatiens balsamina flowers (Klein and Hagen, 1961) and in sunflower cotyledons (Servattaz et al., 1975); whilst gibberellin, auxin and

Figure 43

Anthocyanin levels during turion formation in developing turions and associated mother fronds during incubation in 1 x 10^{-7}M ABA, and during vegetative frond development. Anthocyanin was extracted from the tissue with HCl and determined spectrophotometrically.

S. polyrrhiza (N).

● developing turions

○ mother fronds

developing vegetative fronds

Levels on a gram fresh weight basis:

Developing turions = 80 → 420 μg. g^{-1} (1965 μg. g^{-1})*

Mother fronds = 98 → 54 μg. g^{-1}

Developing vegetative fronds = 78 → 69 μg. g^{-1}

* fully mature turions at day 10

FIGURE 43

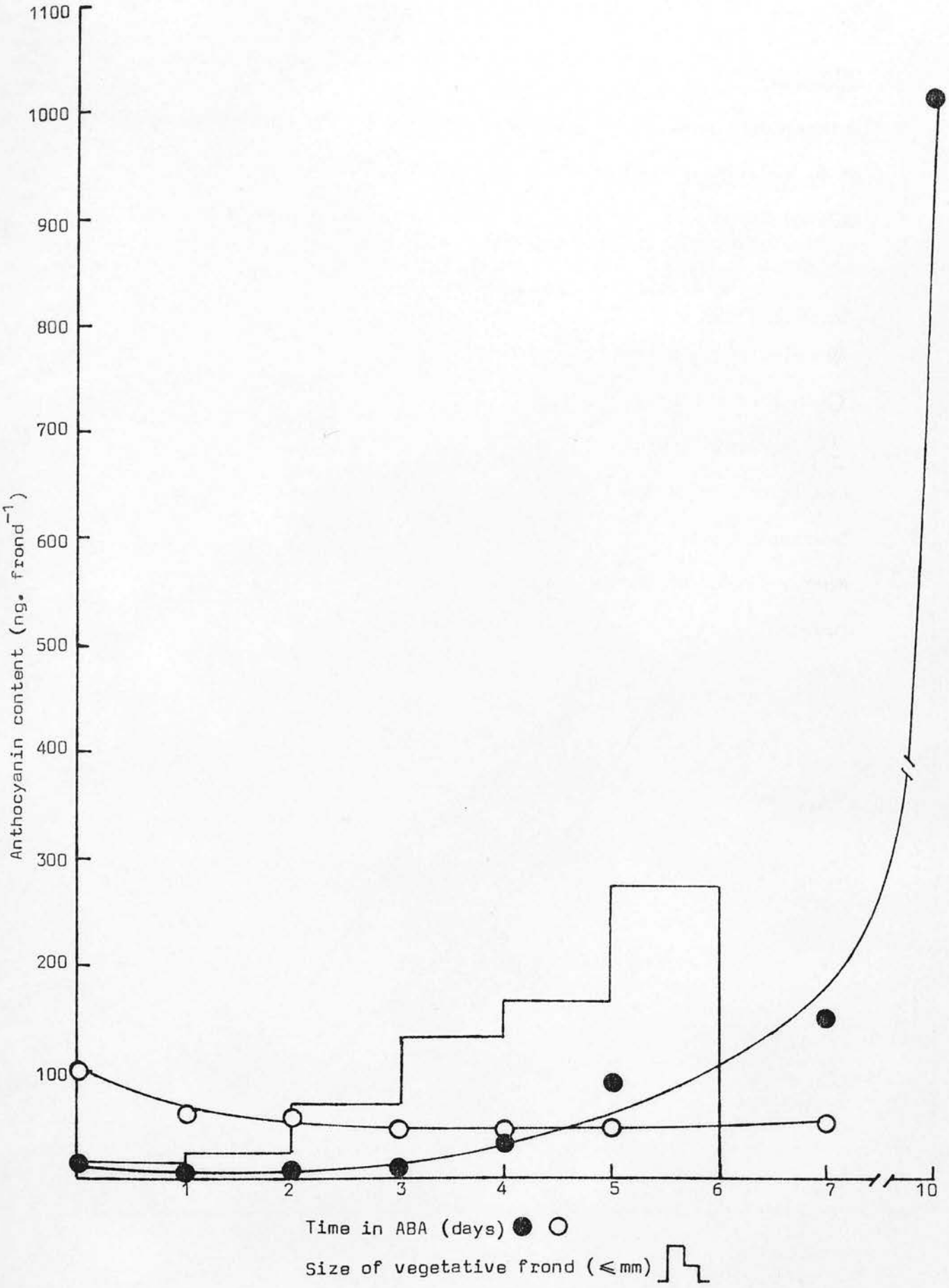

1100
1000
900
800
700
600
500
400
300
200
100
Anthocyanin content (ng. frond^{-1})
1
2
3
4
5
6
7
10
Time in ABA (days)
Size of vegetative frond (≤mm)

Figure 44

Anthocyanin levels during turion formation in the developing turions of S. polyrrhiza (UC) incubated in 5×10^{-7} M ABA.

● ng. frond^{-1}

○ µg. g^{-1}

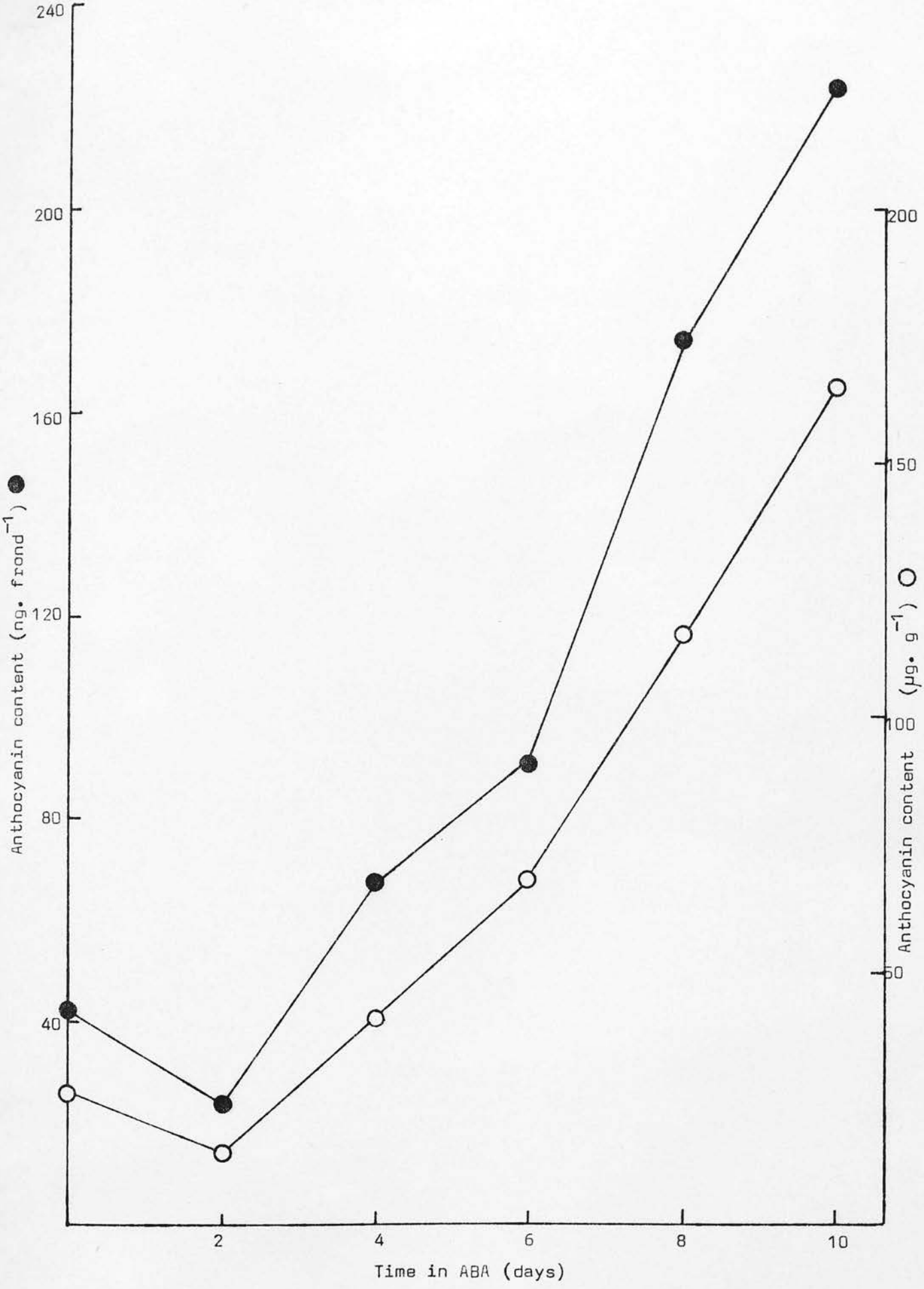

240
200
160
120
80
40
Anthocyanin content (ng. frond^{-1}) ●
200
150
100
50
Anthocyanin content (µg. g^{-1}) ○
2
4
6
8
10
Time in ABA (days)

FIGURE 44

ethylene all appeared to lower the levels or to delay the appearance of anthocyanins in a range of tissues including cell cultures, hypocotyls and stem internodes (Vince, 1968; Constabel et al., 1971; Craker et al., 1971; Kang and Burg, 1973; Gregor, 1974). ABA had no effect (Servattaz et al., 1975). Various responses to ethylene have been noted (Craker, 1975) in which anthocyanin levels were raised if ethylene was present during the lag phase before pigment appearance, but lowered if present during the phase of rapid pigment accumulation. Both gibberellic acid and ABA inhibit the synthesis of anthocyanin in radish, while addition of precursors of anthocyanin synthesis nullifies the inhibition of GA_3 but not of that caused by ABA (Guruprasad and Laloraya, 1980). It was suggested that the inhibition of anthocyanin synthesis by ABA was dependent, not only on a depletion of substrates, but also by the inhibition of a required mRNA species.

In S. oligorrhiza which does not produce turions and in S. polyrrhiza, GA_3 was found to inhibit anthocyanin and flavonoid biosynthesis, but the effect of ABA was not investigated since it was not then known (Furuya and Thimann, 1964). Anthocyanin production by S. oligorrhiza has been studied in detail by Thimann and collaborators since 1949. Preliminary work (Thimann and Edmondson, 1949) showed that anthocyanin accumulated under any condition that reduced the growth rate of the frond and thus resulted in the accumulation of sugars. In growing cultures glucose promoted growth and not anthocyanin production (Thimann, Edmondson and Radner, 1951) while the opposite was true of sucrose. In non-growing cultures however, sucrose, glucose and fructose all promoted

anthocyanin production equally. The authors concluded that all 3 sugars could be utilised directly in anthocyanin synthesis but that glucose was preferentially used for growth.

Henssen (1954) found that sucrose and glucose were both used by S. polyrrhiza for growth in darkness, but that only sucrose promoted both anthocyanin formation and turion production. My data are consistent with the idea that the accumulation of sugars is necessary for the production of pigment, since sugar levels and those of anthocyanin increased dramatically during the development of the turion. Moreover Reznik and Menschick (1969) studying the flavonoids of S. polyrrhiza fronds and turions, found 1 extra anthocyanin and 8 new flavonoid compounds in the turions. The flavonoid pattern of the turion was found to be characterised by compounds with a far higher degree of glycosylation.

Whether anthocyanin accumulation is merely a direct result of increased sugar levels during turion formation or whether ABA plays a more direct role in directing the synthesis of pigment is not known.

4.9 ATP LEVELS DURING TURION FORMATION

RNA and protein synthesis are essential processes for continued plant growth and development. The physiological functions and biochemical processes required for plant growth and development, including RNA and protein synthesis, are driven by energy derived from ATP. The levels of ATP were measured in developing turions and associated mother fronds to see if the inhibition of RNA and protein synthesis (sections 4.11 and 4.12) could be due to reduced

availability of ATP. By analogy with the marked increase in ATP levels during the early germination of seeds (Obendorf and Marcus, 1974; Moreland et al., 1974) and the decrease in ATP levels found in germinating bean axes incubated in ABA (Walbot et al., 1975), the results obtained with S. polyrrhiza were surprising (Fig. 45).

There was a 3 fold increase in the ATP pool size during the development of the turion whether the data were expressed on a frond or fresh weight basis. ABA also increased the ATP content of the associated mother fronds, although to a lesser extent. The rise in the level of soluble sugars could be sufficient to stimulate respiration and ATP content according to Ullrich-Eberius et al. (1978). However, although I have not measured the rate of respiration during turion formation, respiration is reported to be much lower in the turion than the vegetative frond of S. polyrrhiza (Czopek, 1967) and of Wolffia arrhiza (Godziemba-Czyz, 1969b). It thus seems unlikely that the respiration rate would increase during the formation of the turion.

It is more likely that the increased ATP pool size reflects a decreased utilisation of ATP due to the inhibition of the various cellular synthetic processes which occur during turion formation. Firstly there is the observation that tissue ATP levels are unaltered or even rise as protein synthesis is blocked (Cocucci and Marrè, 1973; Lin and Hanson, 1974; Lado et al., 1977); and secondly ABA treatment of L. minor resulted in a slight inhibition of respiration (McLaren and Smith, 1976). ABA induced inhibition of respiration has also been observed in germinating bean axes (Walbot et al., 1975) and germinating lettuce seeds (Bex, 1972c), although

Figure 45

ATP content during turion formation in the developing turions and associated mother fronds of S. polyrrhiza (N) incubated in 1 x 10^{-7}M ABA. ATP was determined by the luciferin-luciferase assay.

● developing turions

○ mother fronds

Actual levels:

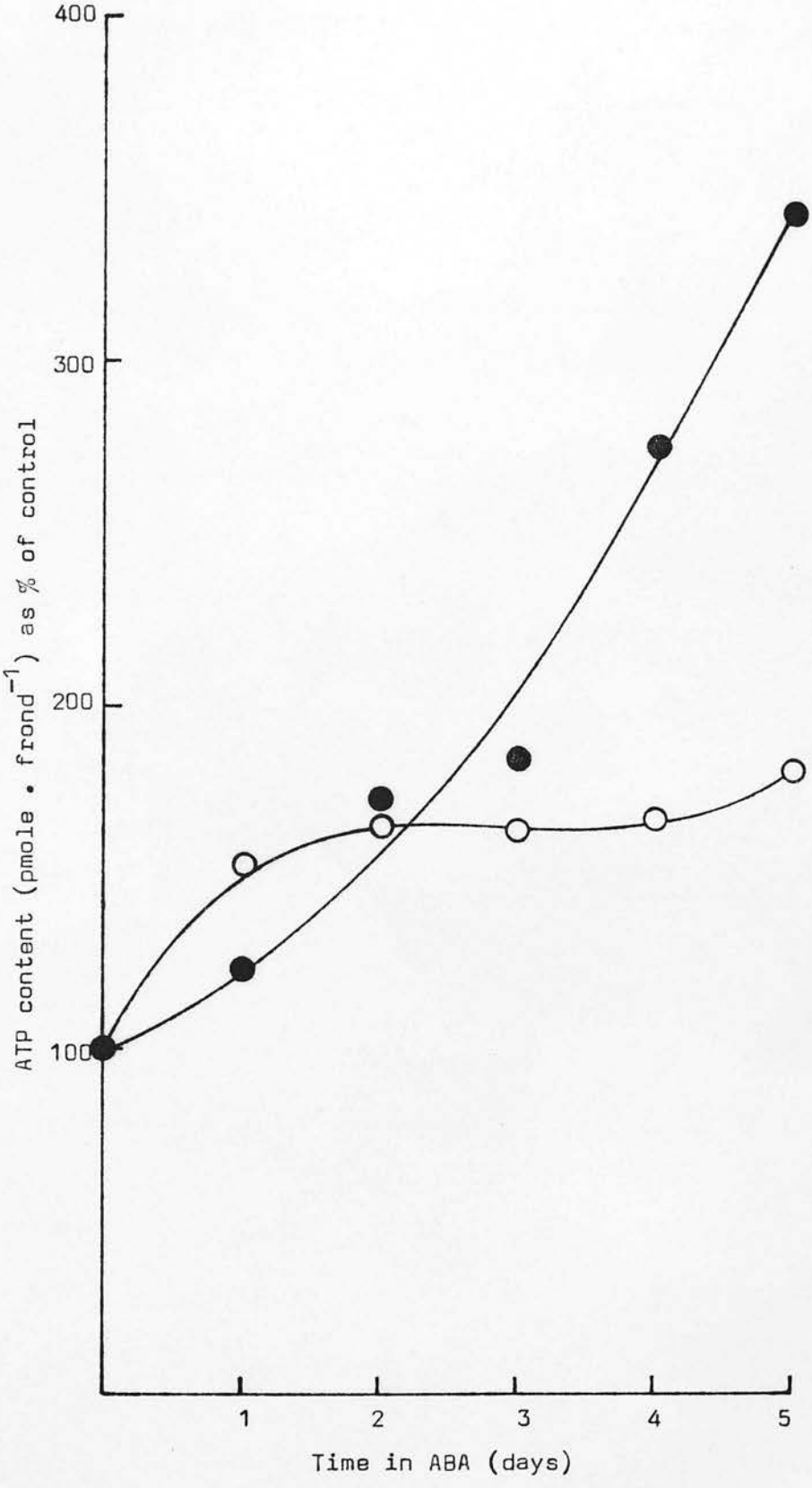
Developing turions = 70·9 → 244·9 pmole. $frond^{-1}$

" " = 378 → 1124 nmole. g^{-1}

Mother fronds = 793 → 1442 pmole. $frond^{-1}$

" " = 245 → 446 nmole. g^{-1}

FIGURE 45


400
300
200
100
ATP content (pmole • frond^{-1}) as % of control
1
2
3
4
5
Time in ABA (days)

this inhibition was neither large nor rapid in character.

4.10 DNA SYNTHESIS DURING TURION FORMATION

In order that investigations into DNA synthesis could be undertaken, the effects of ABA on the uptake of ^{3}H thymidine into developing turions and fronds was investigated. Figure 46 shows the uptake of the precursor into the fronds (as a % of that of untreated controls) as a function of time after application of ABA.

It is clear that the % uptake of ^{3}H thymidine is not only increased in the developing turions but also in the associated mother fronds. The stimulation of uptake is more pronounced in the developing turion than in the mother fronds even when % uptake is expressed on a fresh weight basis, thus correcting for the increase in frond size during turion development. This result is in contrast to the inhibition of ^{3}H thymidine uptake by ABA at 10^{-5} M found by Stewart and Smith (1972). However the stimulation of ^{3}H thymidine uptake reported here was caused by 10^{-7} M ABA, i.e. a turion inducing concentration.

DNA was extracted from developing turions and associated mother fronds incubated in ABA after a 3 hour pulse of ^{3}H thymidine and the specific activity of the DNA was calculated in cpm incorporated. $\mu g\ DNA^{-1} . 10^{6}$ cpm absorbed^{-1} in order to correct for the uptake differences found above. The incorporation of ^{3}H thymidine into DNA is shown in figure 47.

There was no rapid effect of ABA on the specific activity of DNA in the mother fronds as reported for plantlets of S. polyrrhiza incubated at the totally growth inhibiting concentration of 10^{-5} M

Figure 46

The uptake of ^{3}H thymidine into developing turions and associated mother fronds of S. polyrrhiza (N) incubated in 1×10^{-7}M ABA. Tissue was incubated for 3 hours in ^{3}H thymidine, homogenised in ethanol-NaCl and an aliquot taken for counting. Results are expressed as the % uptake. frond^{-1} x 10^{3} as a % of the control (day 0) value.

● developing turions

○ mother fronds

Actual values:

Developing turions % uptake. frond^{-1}. 10^{3} = 0•603 → 2•277

" " % uptake. g^{-1} = 3•2 → 7•9 (a rise of 150%)

Mother fronds % uptake. frond^{-1}. 10^{3} = 8•4 → 16•0

" " % uptake. g^{-1} = 2•6 → 4•9 (a rise of 90%)

FIGURE 46

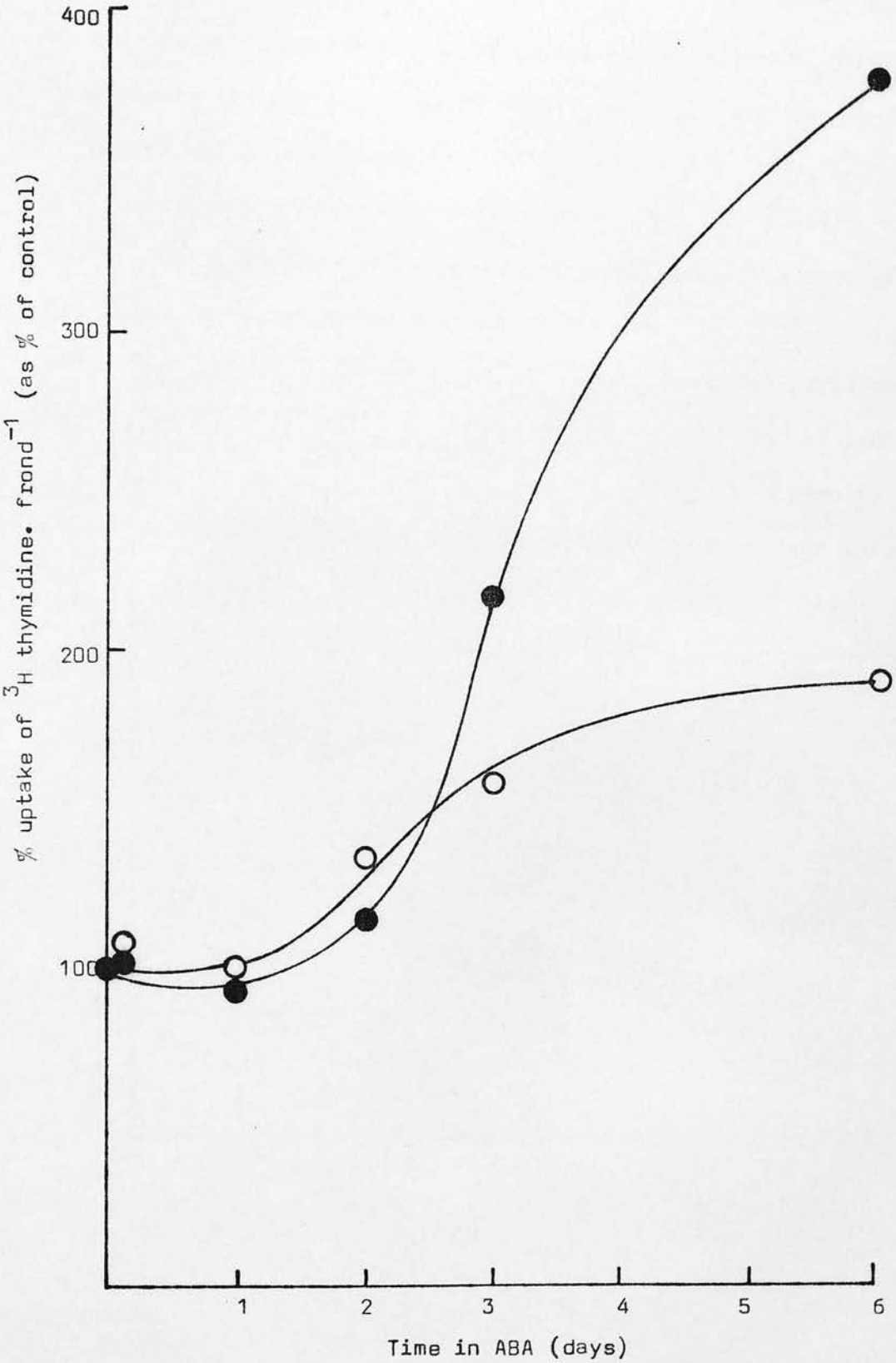

400
300
200
100
% uptake of ^{3}H thymidine. frond^{-1} (as % of control)
1
2
3
4
5
6
Time in ABA (days)

Figure 47

The specific activity of DNA during turion formation in developing turions and associated mother fronds of S. polyrrhiza (N) incubated in 1 x 10^{-7}M ABA. DNA was extracted from the tissue by the method of Guinn after a 3 hour pulse of ^{3}H thymidine and the specific activity of the DNA was calculated in cpm incorporated. $\mu g\ DNA^{-1}$. 10^{6}cpm absorbed^{-1}. DNA was estimated by the diphenylamine assay.

● developing turions

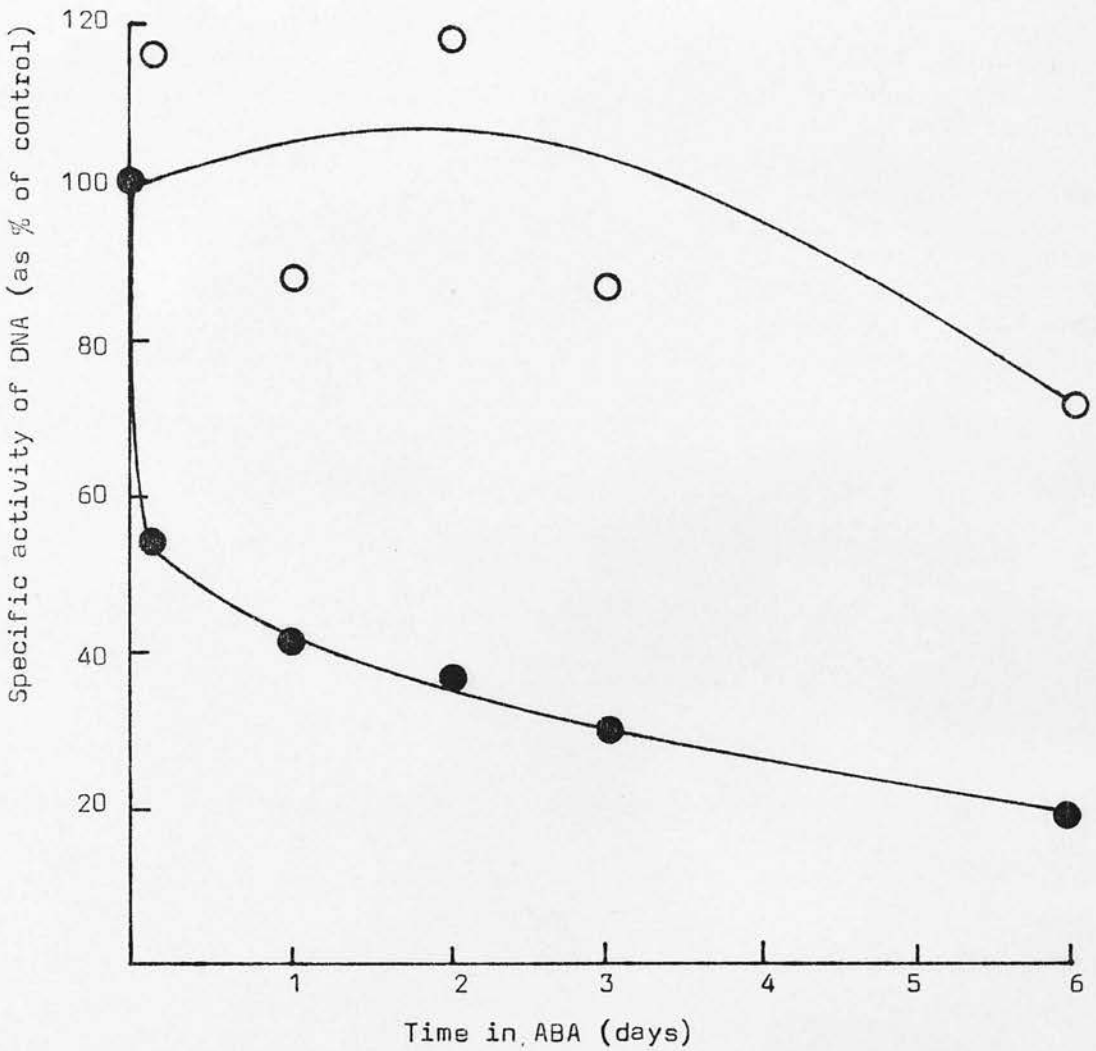
○ mother fronds

Actual values:

Developing turions = 65,073 → 12,750 cpm. $\mu g\ DNA^{-1}$. 10^{6}cpm taken up^{-1}

Mother fronds = 2,191 → 1,562 cpm. $\mu g\ DNA^{-1}$. 10^{6}cpm taken up^{-1}

FIGURE 47


Specific activity of DNA (as % of control)
120
100
80
60
40
20
1
2
3
4
5
6
Time in ABA (days)

ABA (Stewart and Smith, 1972). The specific activity of DNA in the mother fronds was finally lowered after 6 days incubation in ABA by 29% of the activity found in untreated fronds.

The specific activity of DNA in the developing turion however, fell dramatically by 45% within the first 3 hours of ABA application and was inhibited by 80% after 6 days when the turions were nearly mature.

There thus appears to be a far greater sensitivity of the young tissue to ABA. Since the developing turions harvested before the addition of ABA are composed of some cells which are still rapidly dividing, the rapid effect of ABA on the specific activity of DNA probably represents an inhibition of DNA synthesis associated with cell division. However whether the action of ABA is on cell division which leads in turn to inhibition of DNA synthesis, or on DNA synthesis directly is unknown. Since the label used was thymidine, the cultures were regularly monitored for sterility, and the incorporation itself carried out under sterile conditions, it is thus very unlikely that any bacterial DNA synthesis has been measured. The possibility cannot be ruled out however that the label was incorporated into metabolically labile DNA (Pelc, 1968), since this DNA has been found in plants (Bryant et al., 1974). The inhibition of the specific activity of the DNA by ABA may represent reduced turnover of this labile DNA.

There is good evidence however that ABA does inhibit growth and DNA synthesis (or turnover) in radish seedlings (Sarrouy-Balat et al., 1973) and in Spirodela polyrrhiza (Stewart and Smith, 1972). Nevertheless the major part of the evidence suggests that ABA does

not directly affect DNA synthesis, but that the primary target of ABA is on cell division which subsequently leads to inhibition of DNA synthesis. In other tissues work has indicated that DNA synthesis is almost certainly not the primary target of ABA, since there was no apparent correlation between ABA inhibited DNA synthesis and ABA inhibited growth (Chrispeels and Varner, 1967; Walton et al., 1970). This has been shown clearly in dry wheat embryos by Chen and Osborne (1970) who found that protein synthesis commenced from imbibition and was inhibited at 6 hours by ABA, whereas RNA synthesis was not measurable until 12 hours and DNA synthesis until 24 hours. Moreover Haber et al. (1969) have shown that the growth regulatory effect of ABA is still evident in systems where DNA synthesis is excluded.

It is of interest to note that growth inhibition of S. polyrrhiza by 5-fluorodeoxyuridine, a potent inhibitor of DNA synthesis (Rimon and Galun, 1967) did not lead to turion formation (Stewart and Smith, 1972), and indeed turion formation could not and does not occur under conditions of total inhibition of DNA replication.

4.11 RNA SYNTHESIS DURING TURION FORMATION

Figure 48 shows the uptake of ^{3}H uridine into the fronds as a function of time after application of ABA. As with the uptake of ^{3}H thymidine, the % uptake of ^{3}H uridine is increased during the development of the turion and in the associated mother fronds, although to a much lesser extent in both tissues than the stimulation of ^{3}H thymidine uptake. Indeed when the % uptake during the development of the turion is expressed on a fresh weight basis,

Figure 48

The uptake of ^{3}H uridine during turion formation into developing turions and associated mother fronds of S. polyrrhiza (N) incubated in 1 x 10^{-7}M ABA. Tissue was pulse labelled for 6 hours in ^{3}H uridine and homogenised in ethanol-NaCl. An aliquot was taken for counting. Results are expressed as % uptake. frond^{-1}. 10^{3} as a % of the control (day 0) value.

● developing turions

○ mother fronds

Actual values:

Developing turions % uptake. frond^{-1}. 10^{3} = 0·060 → 0·141

" " % uptake. g^{-1} = 0·318 → 0·271 (fall of 15%)

Mother fronds % uptake. frond^{-1}. 10^{3} = 0·814 → 0·946

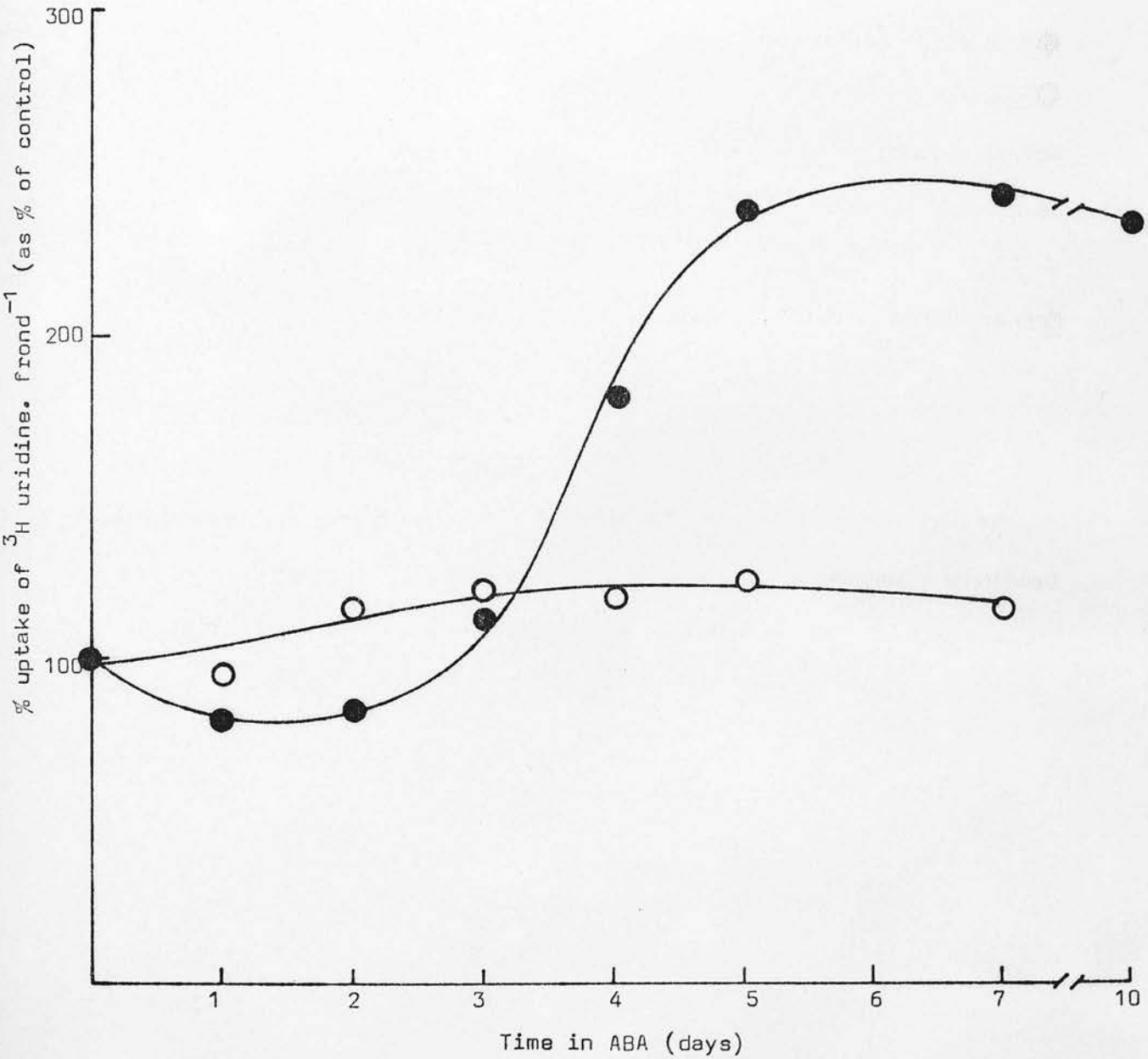
" " % uptake. g^{-1} = 0·252 → 0·292 (rise of 16%)

Developing vegetative fronds % uptake. frond^{-1}. 10^{3} = 0·060 → 1·494 (rise of 2387%)

" " " % uptake. g^{-1} = 0·318 → 0·374 (rise of 18%)

Developing vegetative fronds not shown on figure.

FIGURE 48


300
200
100
% uptake of ^{3}H uridine. frond^{-1} (as % of control)
1
2
3
4
5
6
7
10
Time in ABA (days)

uptake is still stimulated by 106% after 5 days, but is increasingly inhibited as the turion matures. Uptake of ^{3}H uridine is inhibited by 15% in the fully mature turion.

The incorporation of ^{3}H uridine into RNA is shown in figure 49. RNA was extracted from the developing turions and the associated mother fronds incubated in ABA, and additionally from developing vegetative fronds, after a 6 hour pulse of ^{3}H uridine. The specific activity of the RNA was calculated in cpm incorporated. $\mu g\ RNA^{-1}$. 10^{6} cpm absorbed^{-1} in order to correct for the uptake differences.

RNA synthesis was not inhibited in the mother fronds until approximately 4 days in ABA. The final inhibition and time scale of inhibition was very similar to the inhibition of DNA synthesis found with ABA in this tissue. RNA synthesis in the developing turion was however inhibited after 2 days in ABA, the specific activity of RNA in the fully mature turion being 34% of the value found in developing turions before the addition of ABA. However the pattern of total RNA synthesis in the development of the turion in ABA was found to be very similar to that found during the normal development of the vegetative frond, although there is a slightly higher level of RNA synthesis occurring within the turion than in the mature vegetative frond.

Since there is no rapid effect of ABA on the synthesis of RNA in the developing turion it seems very unlikely that ABA affects RNA synthesis directly. A rapid effect of ABA on cell division would lead to the observed sequential inhibition of DNA and RNA synthesis, since the majority of the labelled RNA is probably ribosomal RNA (Stewart and Smith, 1972). A slowing in the rate of RNA synthesis

Figure 49

The specific activity of RNA during turion formation in developing turions and associated mother fronds incubated in 1 x 10^{-7}M ABA; and during the development of the vegetative frond of S. polyrrhiza (N). Tissue was pulse labelled for 6 hours with ^{3}H uridine and RNA was extracted by the method of Guinn. RNA content was determined spectrophotometrically. Results are expressed in cpm incorporated. $\mu g.RNA^{-1}$. 10^{6} cpm absorbed^{-1} as a % of the control (day 0) value.

● developing turions

○ mother fronds

developing vegetative fronds

Actual values:

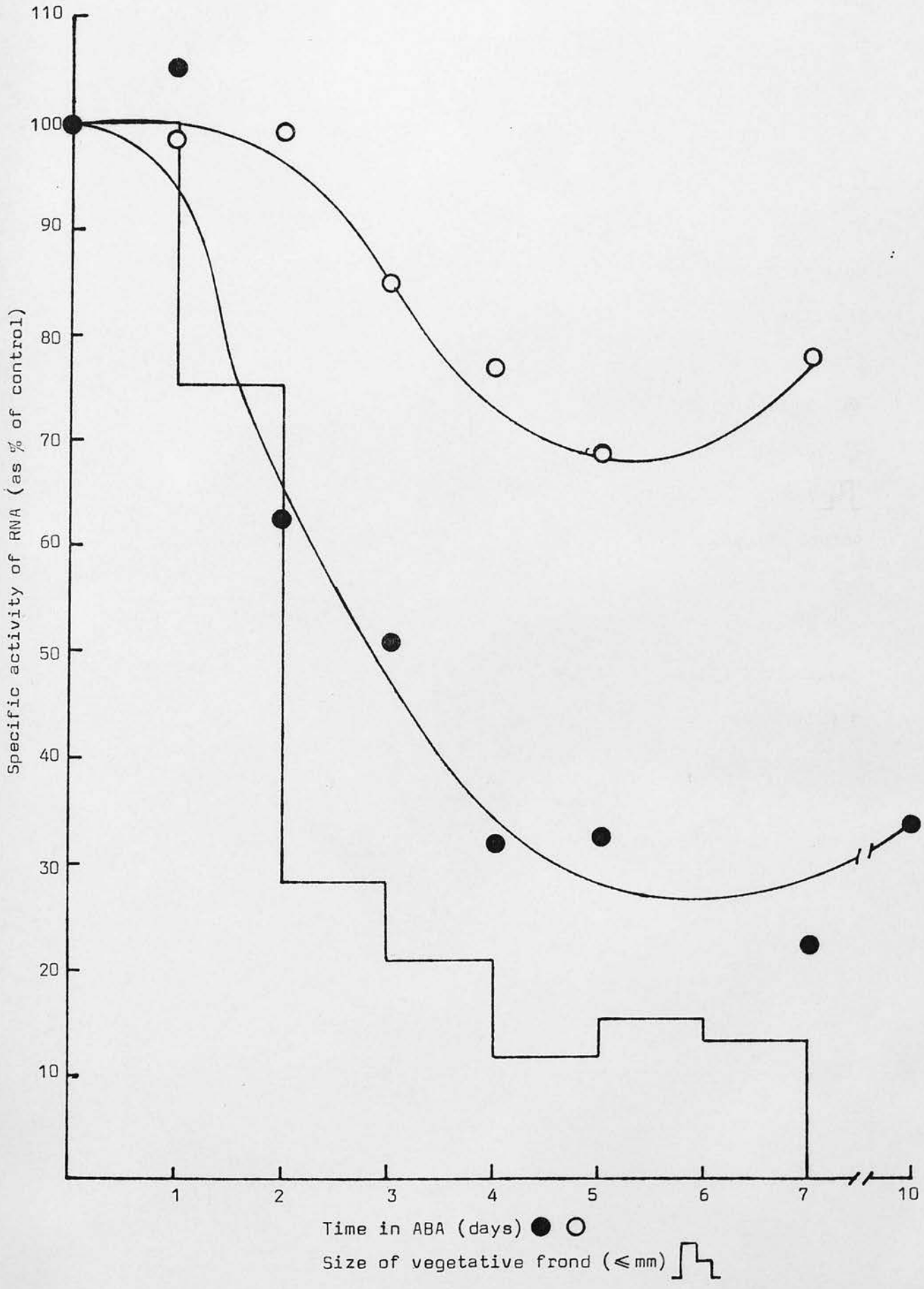
Developing turions = 5401 → 1188 cpm. µg RNA. 10^{6}cpm taken up^{-1} (1860 cpm)*

Mother fronds = 233 → 183 cpm. µg RNA. 10^{6}cpm taken up^{-1}

Developing vegetative fronds = 5284 → 719 cpm. µg RNA. 10^{6}cpm taken up^{-1}

* fully mature turion at day 10

FIGURE 49


110
100
90
80
70
60
50
40
30
20
10
Specific activity of RNA (as % of control)
1
2
3
4
5
6
7
10
Time in ABA (days) ● ○
Size of vegetative frond (≤ mm)

would be expected in the developing turion since it takes 10 days for ABA to induce a fully mature turion, and the cells can be neither dividing nor expanding as rapidly as the cells of the developing fronds which are fully mature within 6 days. As a vegetative frond grows from approximately 1 mm to 2 mm its level of RNA synthesis is decreased by 25%; as a turion develops to ~2 mm the level drops by 77%.

However inhibition of the synthesis of the major RNA species has not been found to enhance or duplicate the effects of ABA (Paranjothy and Wareing, 1971), and indeed in embryonic bean axes growth was found not to be dependent upon rRNA or tRNA synthesis, although ABA reduced the synthesis of these fractions (Walton et al., 1970). 5FU, an inhibitor of RNA synthesis produced inhibition of frond multiplication in S. polyrrhiza without causing turion initiation (Stewart and Smith, 1972). The fact that ABA does not inhibit the synthesis of the major species of RNA in aleurone while stongly inhibiting the synthesis of certain enzymes (Chrispeels and Varner, 1966; Zwar and Jacobsen, 1972), also argues for the contention that while ABA may directly inhibit the synthesis of major RNA species, this is not the RNA of importance to its action, at least initially.

There is evidence that the synthesis or inhibition of minor RNA species, probably mRNA, is involved in ABA action and for this reason mRNA levels were investigated during turion formation (Chapter 5).

4.12 PROTEIN SYNTHESIS DURING TURION FORMATION

The uptake of ^{35}S methionine in developing turions and associated mother fronds is shown in figure 50. The % uptake of ^{35}S methionine decreased with time in the developing turion whether the results were expressed on a frond or fresh weight basis. Although the uptake increased during the development of the vegetative frond, when expressed on a fresh weight basis, there was a decrease in the % uptake as the frond matured similar in extent to that found during the development of the turion. ABA slightly stimulated the uptake of ^{35}S methionine in the associated mother fronds, but only after 6 days.

The specific activity of total protein in the developing turion decreased after 1 day in ABA leading to a 68% inhibition after 6 days (Fig. 51). The decrease in the specific activity of protein during the development of the turion is also very similar to the pattern shown during the maturation of the normal vegetative frond, except for the resumed activity of the fronds between 5 and 6 mm long. This increase could possibly represent the onset of senescence in these fronds, as although some authors have stated that the rate of protein synthesis declines during senescence (Osborne, 1967; Wollgiehn, 1967), there are a number of reports of a maintained or even an increased rate of amino acid incorporation (Atkin and Srivastava, 1970; Hedley and Stoddart, 1972; Spencer and Titus, 1972; Brady et al., 1974).

Protein synthesis in the mother fronds also declines with time of incubation in ABA, although to a smaller extent than the

Figure 50

The uptake of ^{35}S methionine during turion formation in developing turions and associated mother fronds incubated in 1×10^{-7}M ABA of S. polyrrhiza (N). Tissue was pulse labelled for 3 hours, homogenised in NaOH and an aliquot taken for counting. Results are expressed as % uptake. frond^{-1}. 10^3 as a % of the control (day 0) value.

● developing turions

○ mother fronds

Actual values:

Developing turions % uptake. frond. 10^3 = 0·645 → 0·310

" " % uptake. g^{-1} = 3·44 → 1·11 (fall of 68%)

Mother fronds % uptake. frond^{-1}. 10^3 = 1·884 → 2·711

" " % uptake. g^{-1} = 0·58 → 0·84 (rise of 45%)

Developing vegetative fronds % uptake. frond^{-1}. 10^3 = 0·557 → 5·156 (rise of 826%)

" " " % uptake. g^{-1} = 2·97 → 1·29 (fall of 57%)

Developing vegetative fronds not shown on figure.

FIGURE 50

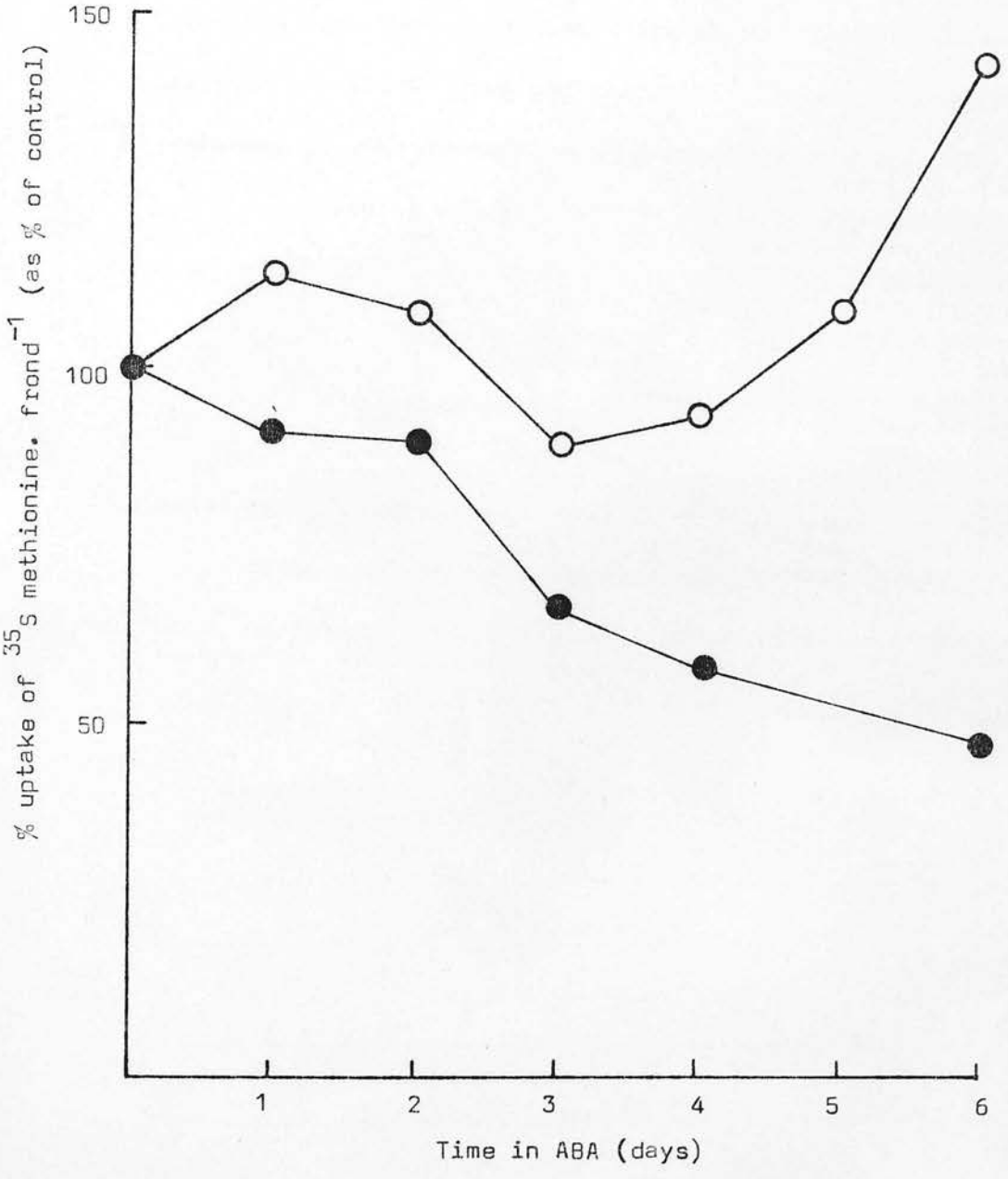

150
100
50
% uptake of ^{35}S methionine. frond^{-1} (as % of control)
1
2
3
4
5
6
Time in ABA (days)

Figure 51

The specific activity of total protein during turion formation in developing turions and associated mother fronds of S. polyrrhiza (N) incubated in 1 x 10^{-7}M ABA; and during the development of the vegetative fronds. Tissue was pulse labelled for 3 hours in ^{35}S methionine, homogenised in NaOH, and protein was TCA precipitated and assayed by the Lowry method. Radioactivity associated with the protein was determined by counting the NaOH extract before Lowry assay. Results are expressed in cpm incorporated. μg protein.$^{-1}$ 10^6 cpm absorbed^{-1} as a % of the control (day 0) value.

● developing turions

○ mother fronds

developing vegetative fronds

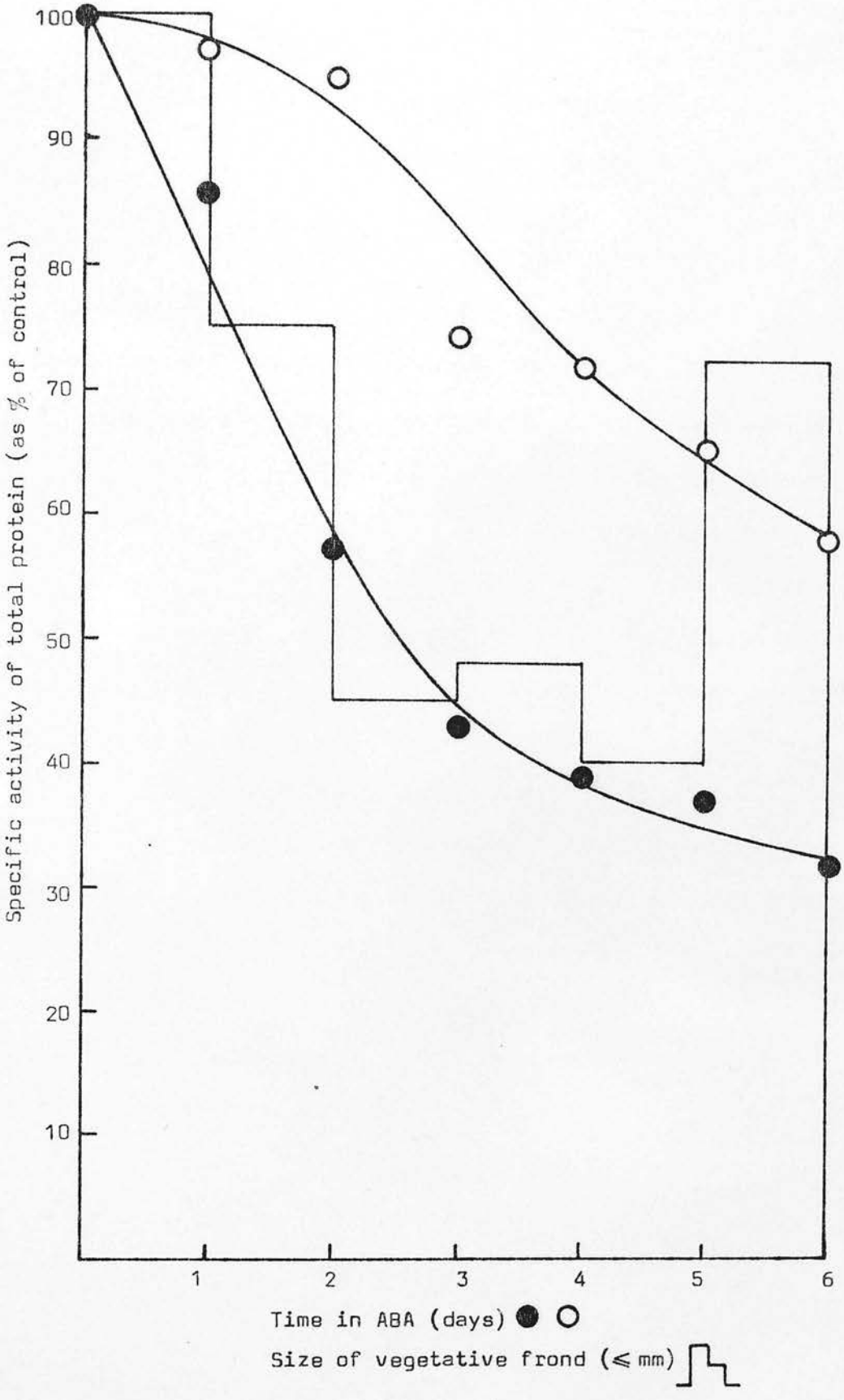
Actual values:

Developing turions = 1251 → 406 cpm. μg protein.$^{-1}$ 10^6 cpm taken up^{-1}

Mother fronds = 74 → 43 cpm. μg protein^{-1}. 10^6 cpm taken up^{-1}

Developing vegetative fronds = 821 → 599 cpm. μg protein^{-1}. 10^6 cpm taken up^{-1}

FIGURE 51


100
90
80
70
60
50
40
30
20
10
Specific activity of total protein (as % of control)
1
2
3
4
5
6
Time in ABA (days)
Size of vegetative frond (≤ mm)

inhibition found during the development of the turion. Once again the 2 types of tissue appear to have a differential sensitivity to ABA.

Differential effects in protein synthesis were studied by repeating these experiments for the different fractions of protein; homogenate (Fig. 52), soluble (Fig. 53), Fraction 1 protein (Fig. 54) and insoluble protein (Fig. 55). Fraction 1 protein was studied as one representative of a soluble protein. The decline in the specific activity of homogenate, soluble and F1 protein fractions during the development of the turion followed similar patterns; homogenate protein synthesis declining by 46%, soluble protein by 36% and F1 protein by 74% within 1 day of ABA application. Insoluble protein synthesis however was not inhibited at all after 1 day, although after 7 days in ABA the extent of inhibition was similar to that found in the other fractions.

The overall inhibition of total protein synthesis during the development of the turion was therefore due to an inhibition of all fractions of cellular protein, although this inhibition was differential i.e. F1 protein synthesis declined more rapidly than soluble protein generally, and soluble protein declined more rapidly than membrane bound protein. However in such overall labelling studies, an overall inhibition of protein synthesis by ABA might result in a preferential decrease in the specific activity of proteins with short lives i.e. fast rates of turnover.

Since the low concentration of ABA used in this experiment also reduced the specific activity of all protein fractions in the mother fronds, although to a lesser extent than during the development of

Figure 52

The specific activity of homogenate protein during turion formation in developing turions and mother fronds incubated in 1 x 10^{-7}M ABA; and during the development of the vegetative frond of S. polyrrhiza (N). Tissue was homogenised in grinding buffer and protein was estimated by the Lowry and Bradford methods. Results are expressed as cpm incorporated. µg protein^{-1}. 10^6 cpm absorbed^{-1} as a % of the control (day 0) value.

● developing turions

○ mother fronds

developing vegetative fronds

Actual values:

Developing turions = 8,751 → 733 cpm. µg protein^{-1}. 10^6cpm absorbed^{-1}

Mother fronds = 1,096 → 1,594 cpm. µg protein^{-1}. 10^6cpm absorbed^{-1}

Developing vegetative fronds = 8,346 → 1,519 cpm. µg protein^{-1}. cpm absorbed^{-1}

FIGURE 52

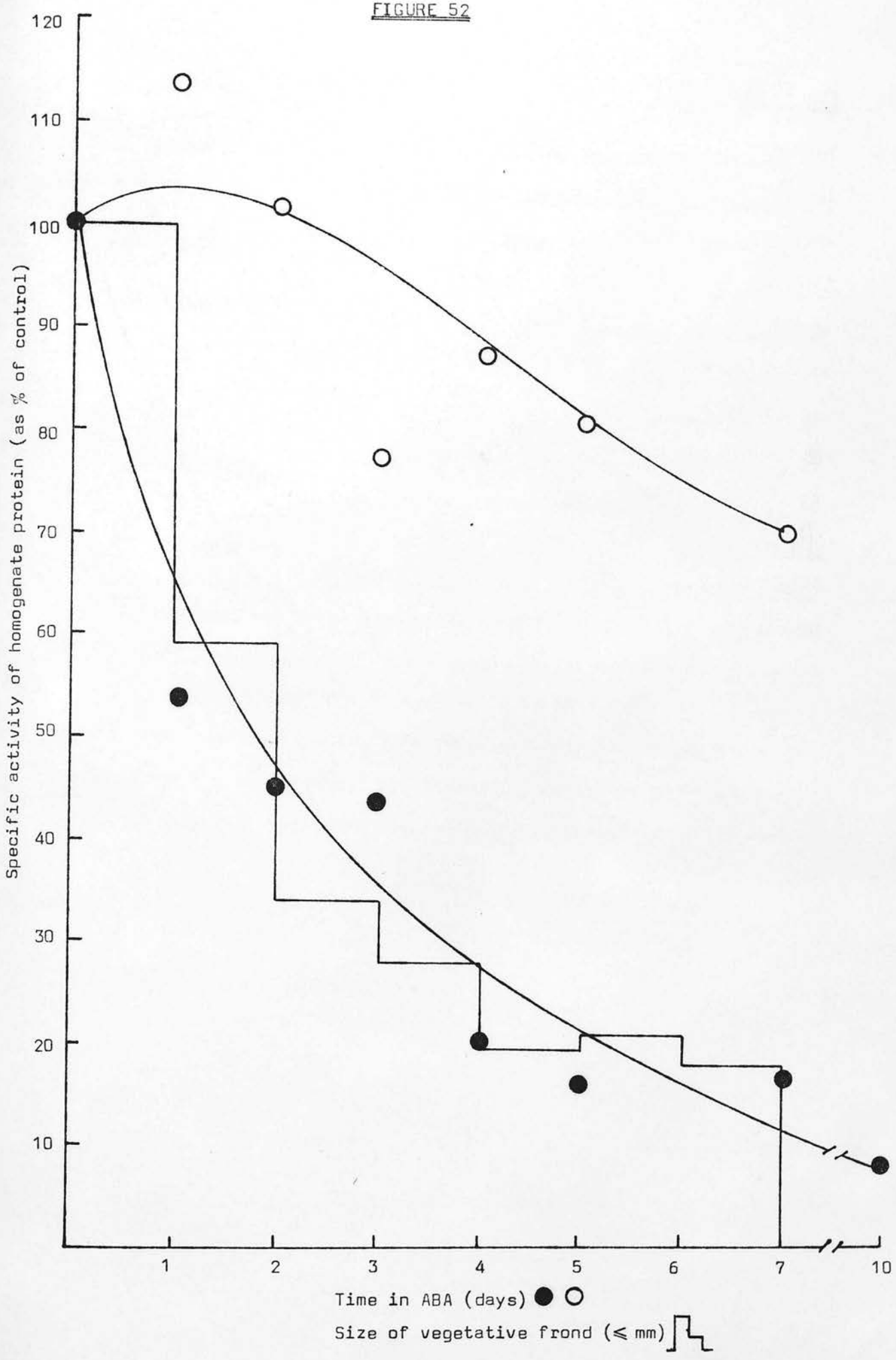

120
110
100
90
80
70
60
50
40
30
20
10
Specific activity of homogenate protein (as % of control)
1
2
3
4
5
6
7
10
Time in ABA (days)
Size of vegetative frond (≤ mm)

Figure 53

The specific activity of soluble protein during turion formation in the developing turions and associated mother fronds incubated in 1×10^{-7}M ABA; and in the developing vegetative frond of S. polyrrhiza (N).

● developing turions

• developing turions*

○ mother fronds

o mother fronds**

developing vegetative fronds

Actual values:

Developing turions = 3,554 → 707 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}
(1,656 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1})*

Mother fronds = 467 → 335 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}
(330 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1})**

Developing vegetative fronds = 3,509 → 372 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}

* fronds ≤ 0·7 mm harvested from plantlets incubated in 1×10^{-5}M ABA

** mother fronds harvested from plantlets incubated in 1×10^{-5}M ABA

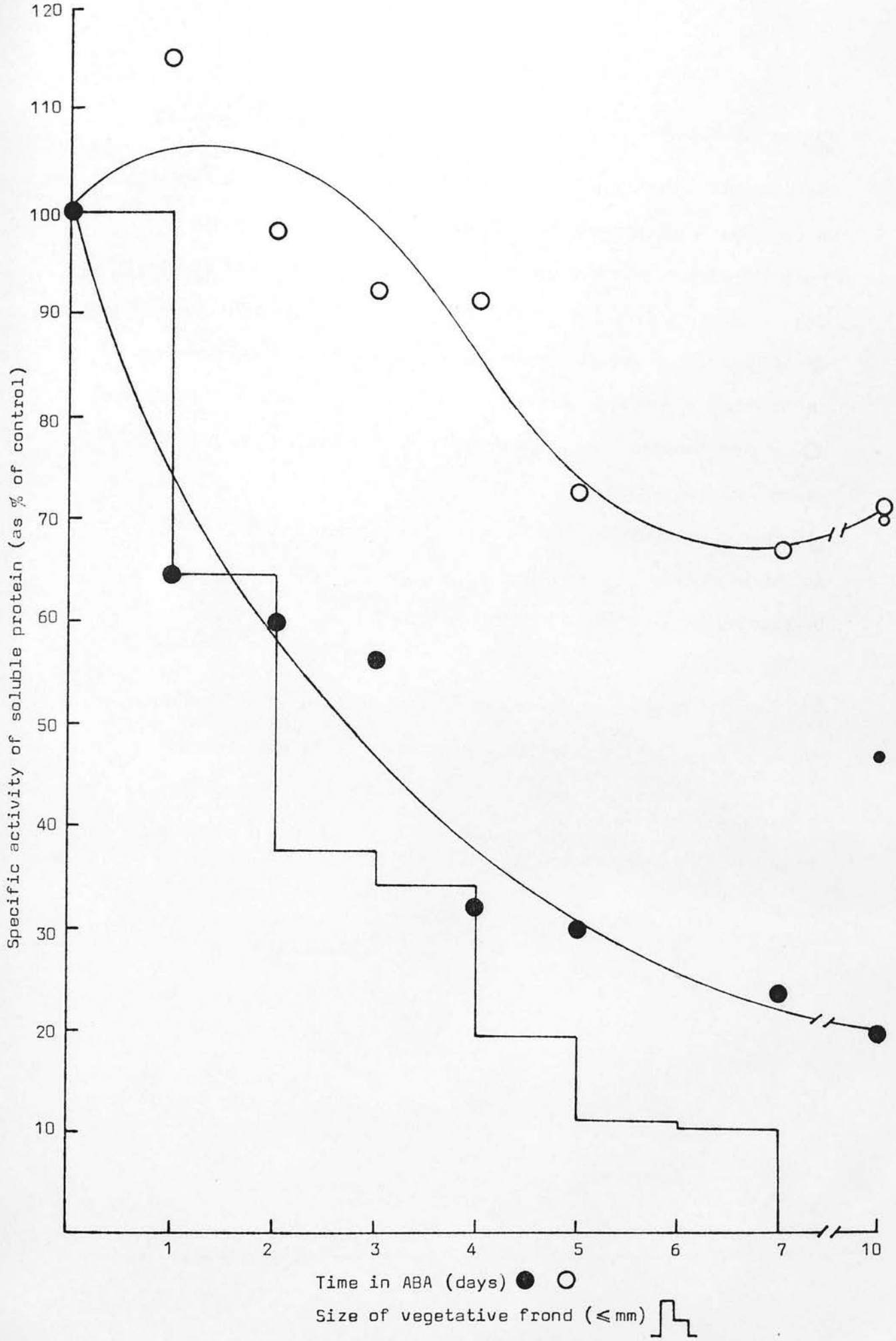

120
110
100
90
80
70
60
50
40
30
20
10
Specific activity of soluble protein (as % of control)
1
2
3
4
5
6
7
10
Time in ABA (days)
Size of vegetative frond (≤ mm)

FIGURE 53

Figure 54

The specific activity of Fraction 1 protein during turion formation in developing turions and associated mother fronds incubated in 1×10^{-7}M ABA; and in the developing vegetative fronds of S. polyrrhiza (N). Tissue was labelled with ^{35}S methionine and protein was estimated by non-denaturing gel elctrophoresis. Radioactivity was measured in the band corresponding to Fraction 1 protein. Results are expressed as cpm incorporated . μg protein^{-1}. 10^6cpm absorbed^{-1} as a % of the control (day 0) value.

● developing turions

○ mother fronds

developing vegetative fronds

Actual values:

Developing turions = 24,021 → 958 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}

Mother fronds = 1,132 → 436 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}

Developing vegetative fronds = 20,694 → 1,138 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}

FIGURE 54

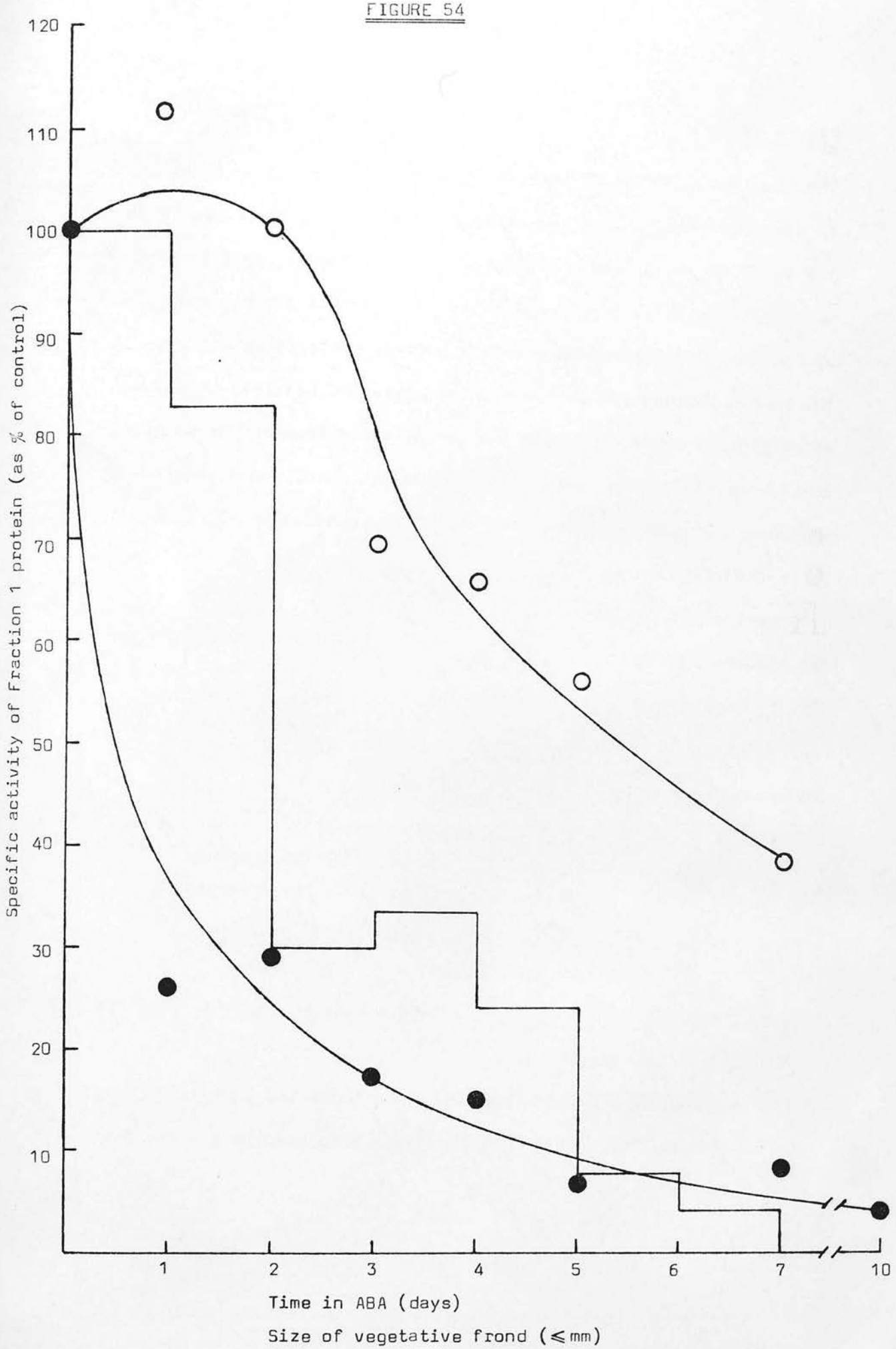

120
110
100
90
80
70
60
50
40
30
20
10
Specific activity of Fraction 1 protein (as % of control)
1
2
3
4
5
6
7
10
Time in ABA (days)
Size of vegetative frond (≤ mm)

Figure 55

The specific activity of insoluble protein during turion formation in the developing turion and associated mother fronds incubated in 1×10^{-7}M ABA; and during the development of the vegetative frond of S. polyrrhiza (N). Tissue was pulse labelled for 3 hours with ^{35}S methionine, homogenised in grinding buffer, centrifuged and the pellet was assayed for protein by the Lowry and Bradford methods. Radioactivity associated with the pellet was determined by counting the protein extracts. Results are expressed as cpm incorporated. ug protein^{-1}. 10^6 cpm absorbed^{-1} as a % of the control(day 0) value.

● developing turions

• developing turions*

○ mother fronds

o mother fronds**

 developing vegetative frond

Actual values:

Developing turion = 1,193 → 228 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}
(462 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1})ǂ
(808 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1})*

Mother fronds = 420 → 270 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}
(240 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1})**

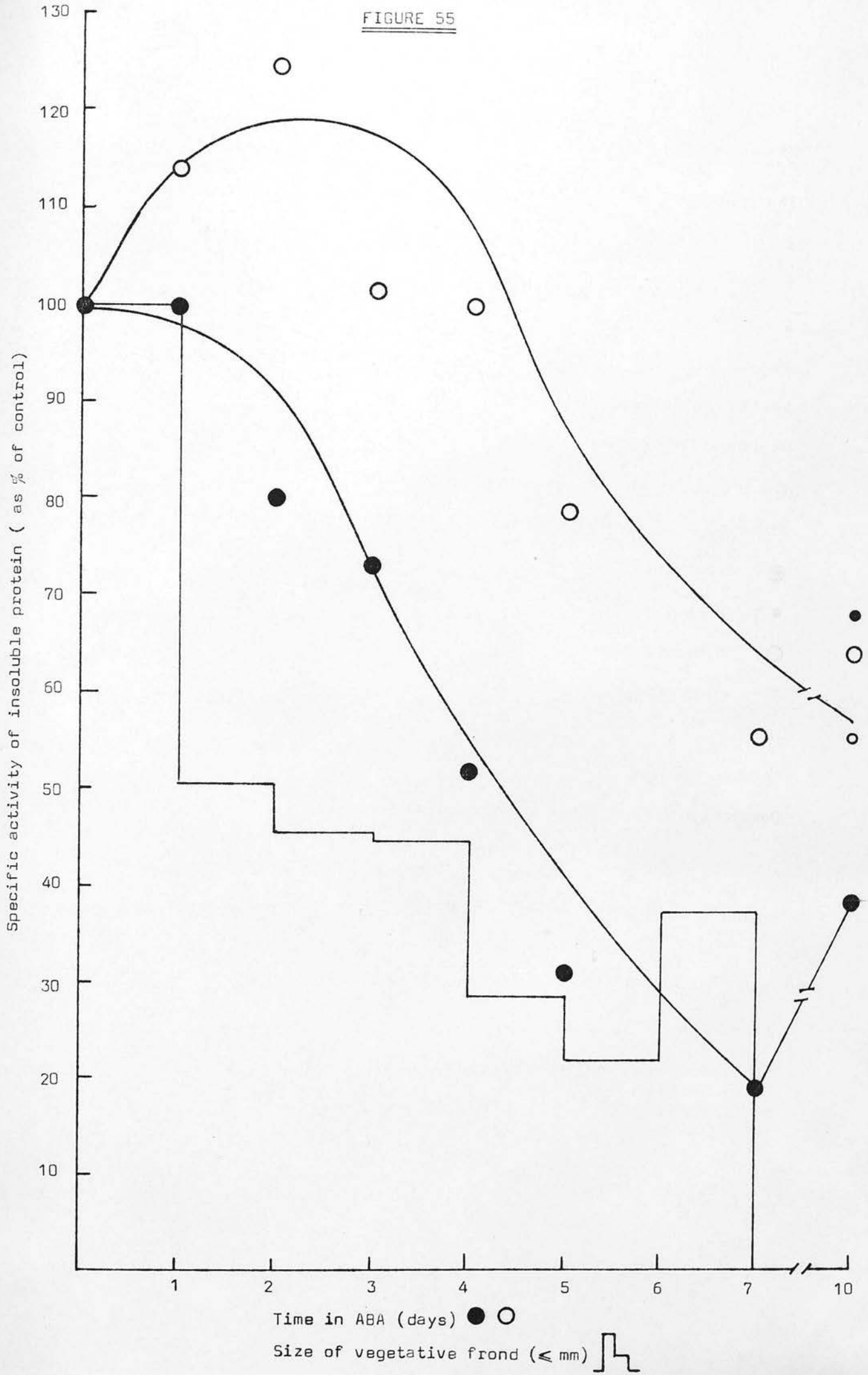
Developing vegetative frond = 1,132 → 424 cpm. μg protein^{-1}. 10^6cpm absorbed^{-1}

ǂ fully developed turions at day 10

* fronds ≤0·7 mm harvested from plantlets incubated in 1×10^{-5}M ABA

** mother fronds harvested from plantlets incubated in 1×10^{-5}M ABA

FIGURE 55


130
120
110
100
90
80
70
60
50
40
30
20
10
Specific activity of insoluble protein (as % of control)
1
2
3
4
5
6
7
10
Time in ABA (days) ● ○
Size of vegetative frond (≤ mm)

the turion, this led to considering if a decline in protein synthesis was directly causal in the production of turions by ABA. This hypothesis seemed very unlikely in view of the fact that inhibitors of protein synthesis such as cyclohexamide and chloramphenicol both produced inhibition of frond multiplication without causing turion formation (Stewart and Smith, 1972). I have examined this further by using azetidine-2-carboxylic acid, a proline analogue, which does not interfere with protein assembly, but by being incorporated instead of proline results in proteins which are ineffective as enzymes.

The effect of 2-azetidine-2-carboxylic acid on the growth and turion production of S. polyrrhiza is shown in figure 56. At 10^{-5} M azetidine, growth was completely inhibited and the fronds appeared to be dead after 7 days. However between 10^{-6} M and 10^{-8} M azetidine the growth of the fronds was inhibited to a similar extent to that shown with 10^{-8} M ABA alone. In conjunction with ABA, azetidine had no additional growth inhibitory effect except at 10^{-7} M ABA which induces the formation of turions. Azetidine depressed turion production slightly between 10^{-6} M and 10^{-8} M, but did not induce the formation of turions either alone or in conjuction with a non-turion forming concentration of ABA.

It seems likely therefore that while there is a general inhibition of protein synthesis during the development of the turion, the production of the turion probably relies on the specific decline of the activities of certain enzymes and/or the production of new ones.

The variation in tissue sensitivity is highlighted by the fact

Figure 56

The effect of 2-azetidine-2-carboxylic acid on the growth rate and turion production of S. polyrrhiza (N). Fronds were incubated in various combinations of ABA and azetidine and left for 10 days. Fronds and turions were then counted, and the growth rate calculated as in Fig.3.

■ no ABA

○ 10^{-8}M ABA

● 10^{-7}M ABA

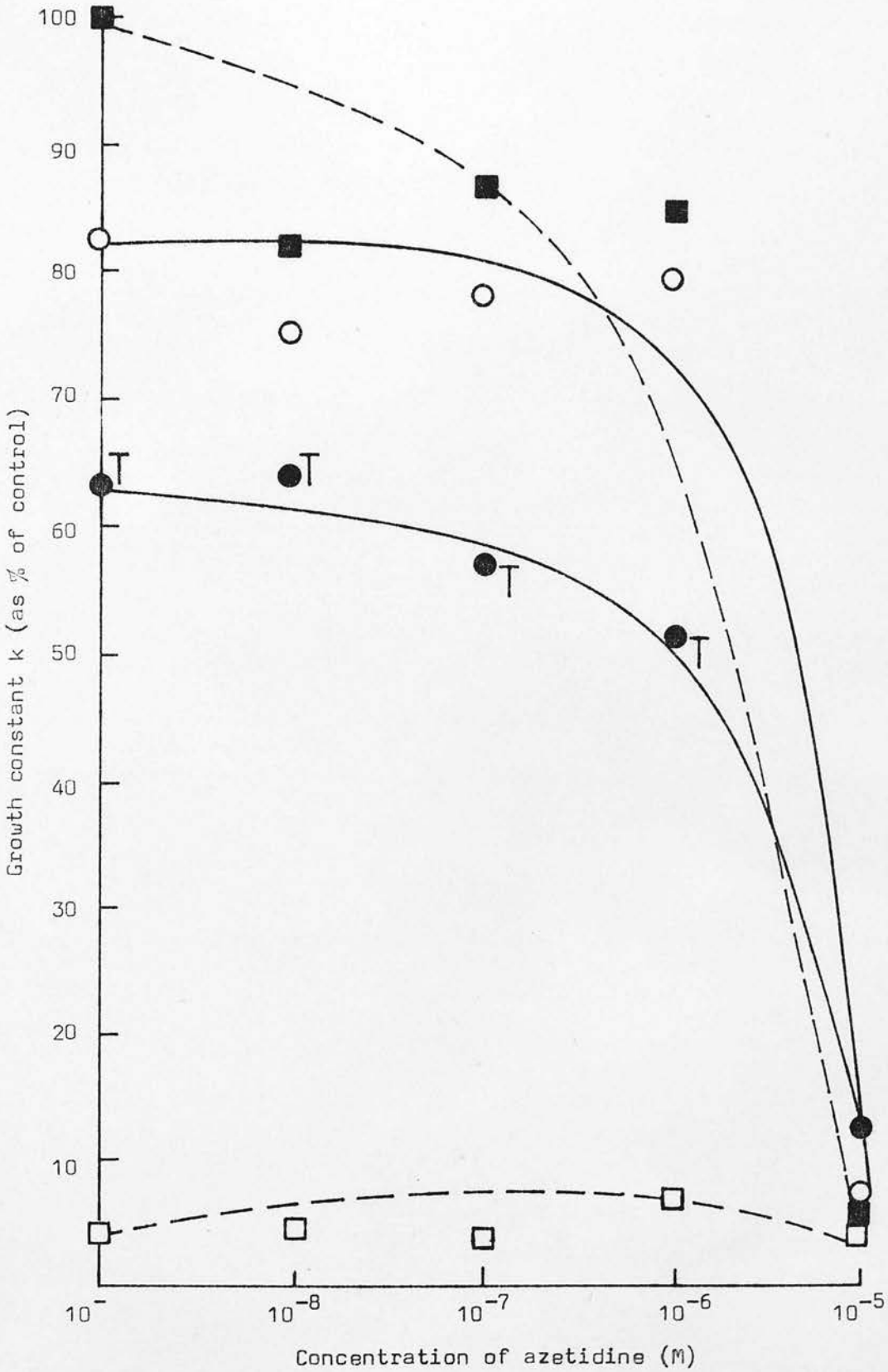
□ 10^{-5}M ABA

T Turions formed

At 10^{-7}M turions were produced and the turions produced as a % of the total number of new fronds formed is shown below:

no azetidine = 46%; 10^{-8}M azetidine = 32%; 10^{-7}M azetidine = 35%; 10^{-6}M azetidine = 38%; 10^{-5}M azetidine = 0% (all fronds dead).

FIGURE 56


100
90
80
70
60
50
40
30
20
10
Growth constant k (as % of control)
T
T
T
T
10⁻
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
Concentration of azetidine (M)

that whereas a completely growth inhibiting and non-turion forming concentration of ABA (10^{-5} M) resulted in an inhibition of protein synthesis in the mother fronds similar to that found with 10^{-7} M ABA, the specific activity of very small fronds ($\leqslant$0·7 mm) incubated for 10 days in 10^{-5} M ABA were not inhibited to the same extent as was the fully mature turion after the same period in 10^{-7} M ABA (Figs. 53 and 55). It therefore becomes difficult to argue that high concentrations of ABA do not result in turion formation because protein synthesis is completely inhibited.

It is important to realise however that data obtained from such labelling studies may not truely reflect protein synthesis. There are many ways (Kuraishi, 1973) in which radioactivity from amino acids might be preferentially detected or lost in protein other than by direct stimulation or inhibition of protein biosynthesis. Alterations in specific activity of amino acid pools, differential incorporation of amino acids and differential metabolism of amino acids might all lead to false conclusions about the effect of plant growth regulators on protein synthesis.

However during the development of the turion it has been shown that the decline in the specific activity of protein correlates also with the decline in the amount of protein present in the developing turion. Nevertheless it was felt necessary to examine the subunit composition of the proteins that were being synthesised during the development of the turion by SDS-polyacrylamide gel electrophoresis and autoradiography. This approach was used to investigate whether there was a selective inhibition of the synthesis of particular proteins.

CHAPTER 5

RESULTS AND DISCUSSION

THE MOLECULAR BASIS OF TURION FORMATION

5.1 IN VIVO LABELLING OF PROTEINS DURING TURION FORMATION

Stained gels of the proteins present during the formation of turions and in the mother fronds associated with the turions indicated that the inhibition of protein synthesis found during the development of the turion in ABA was also accompanied by selective changes in the polypeptide composition of the soluble protein found during the different stages of turion formation. However stained gels can provide only limited information, and with the very small amounts of protein available for study, this technique proved very insensitive. After this work was completed a silver stain technique was described (Switzer et al., 1979) which is far more sensitive than Coomassie blue. Nevertheless stained gels do not provide information on the synthesis of particular protein species.

Although soluble protein levels decreased during turion formation, it was the inhibition of protein synthesis which was particularly striking; and for this reason developing turions, developing fronds and mother fronds incubated in ABA were pulse labelled with ^{35}S methionine, their proteins extracted and analysed by 2-dimensional gel elctrophoresis. The labelled proteins were detected by autoradiographic or fluorographic techniques.

Because of the inhibition of protein synthesis which occurs during turion formation and the varying extent of isotope uptake, equal amounts of protein applied to polyacrylamide gels did not result in the application of equal amounts of radioactivity. This had to be borne in mind when interpreting the results of the experiment in terms of whether the synthesis of a single protein species increases or decreases during turion formation. For this

reason, only large changes which could not be accounted for by changes in the total level of radioactivity present were noted. In practice, this is made less difficult by the observation of overall changes in the total pattern relationship of one protein to another.

Figure 57 shows the soluble proteins synthesised during the development of the turion and figure 58 those associated with the mother fronds. The pattern of proteins synthesised during the normal development of the vegetative frond is shown in figure 59. A complete list of the major soluble polypeptides present in the developing turion is shown in table 8, along with their approximate molecular weights and isoelectric points, while table 9 shows the major changes occurring in the associated mother fronds.

The label in the majority of proteins decreased or disappeared during turion formation, whilst a few showed increased synthesis and others appeared de novo. The most interesting and striking pattern differences are discussed below.

Proteins 7 and 8

The radio-label in these proteins fluctuates widely and rapidly during turion formation. Their synthesis is greatly enhanced after 1 day in ABA, and again 3 days after ABA addition. After 4 days in ABA however their synthesis is extremely reduced , and by day 7 they are barely detectable (Fig. 57). Since they do fluctuate so readily they might possibly have some important regulatory role in turion formation. These proteins also fluctuate in the mother fronds associated with the developing turions, although no decrease in their label occurs as in the developing turions (Fig. 58). The decrease in label of these 2 proteins seems likely to be directly related to

Figure 57

Pattern of *in vivo* labelled proteins during the development of the turion of *S. polyrrhiza* (N) in 1 x 10^{-7}M ABA. Tissue was pulse labelled for 3 hours with ^{35}S methionine and soluble protein was extracted, and loaded onto 2-dimensional polyacrylamide gels. Equal amounts of soluble protein were applied to each gel (50 µg). Labelled proteins were visualised by autoradiography.

a) day 0 (untreated) 92,000 cpm

b) day 1 76,000 cpm

c) day 2 41,000 cpm

d) day 3 38,000 cpm

e) day 4 39,000 cpm

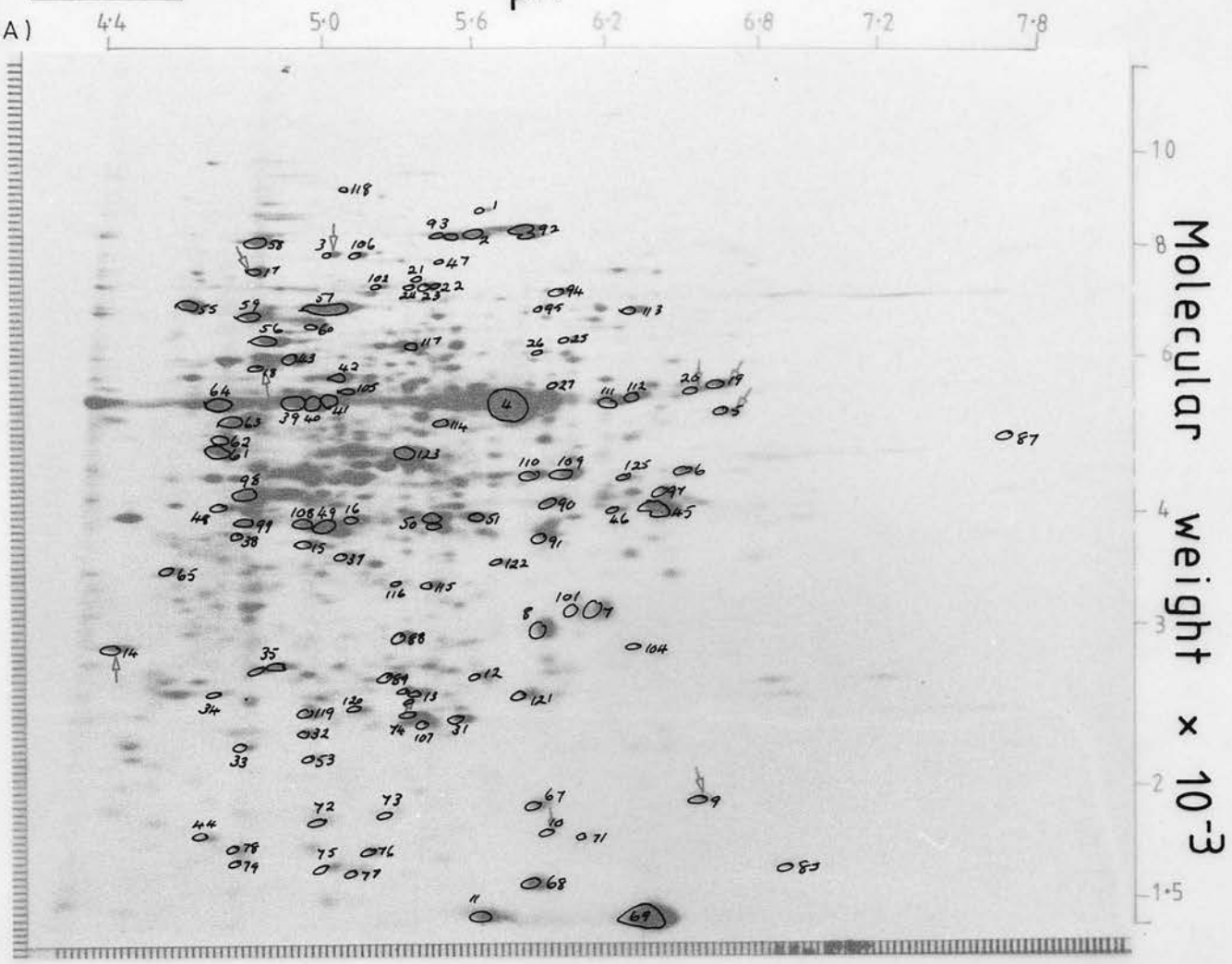
f) day 5 40,000 cpm

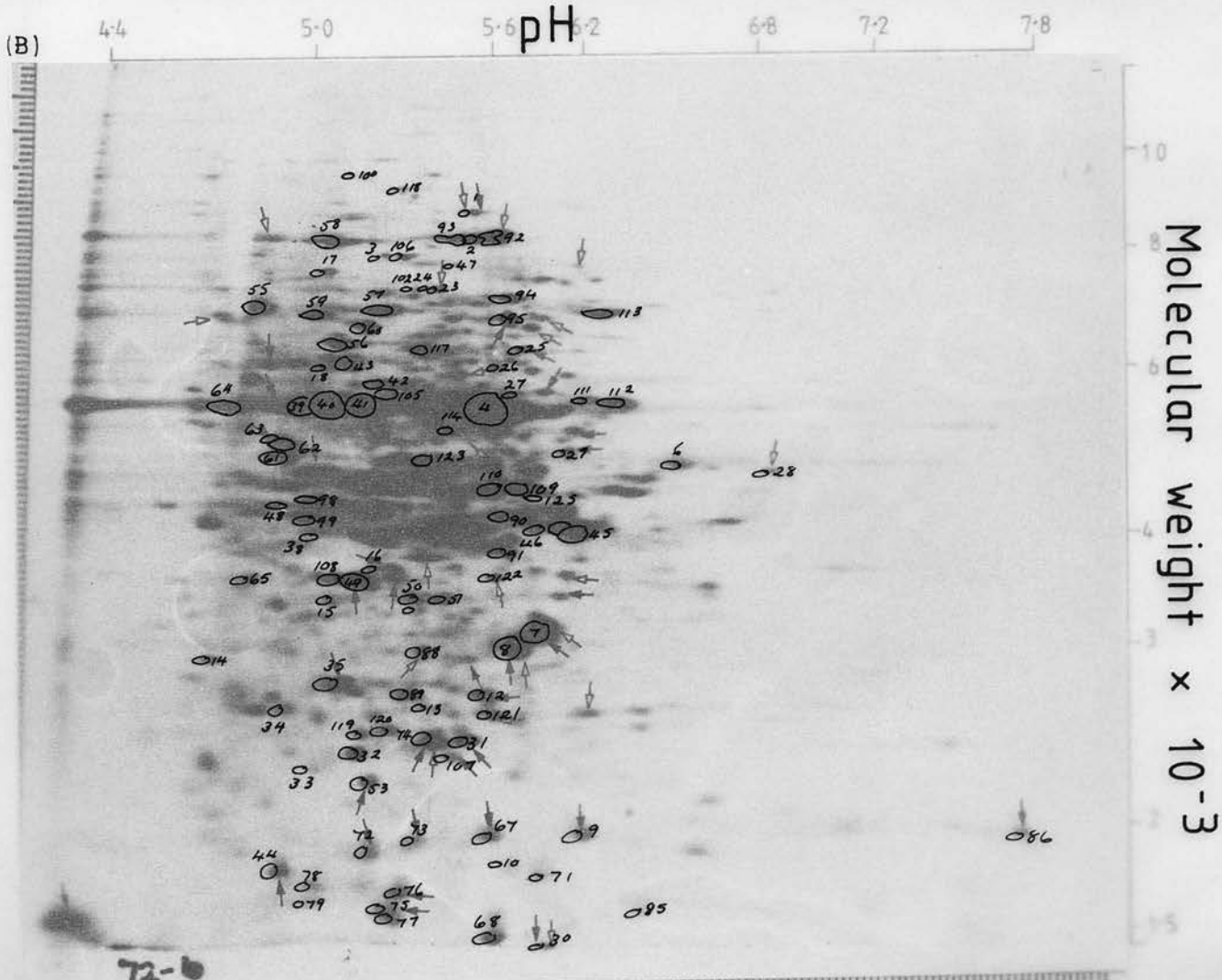
g) day 7 52,000 cpm

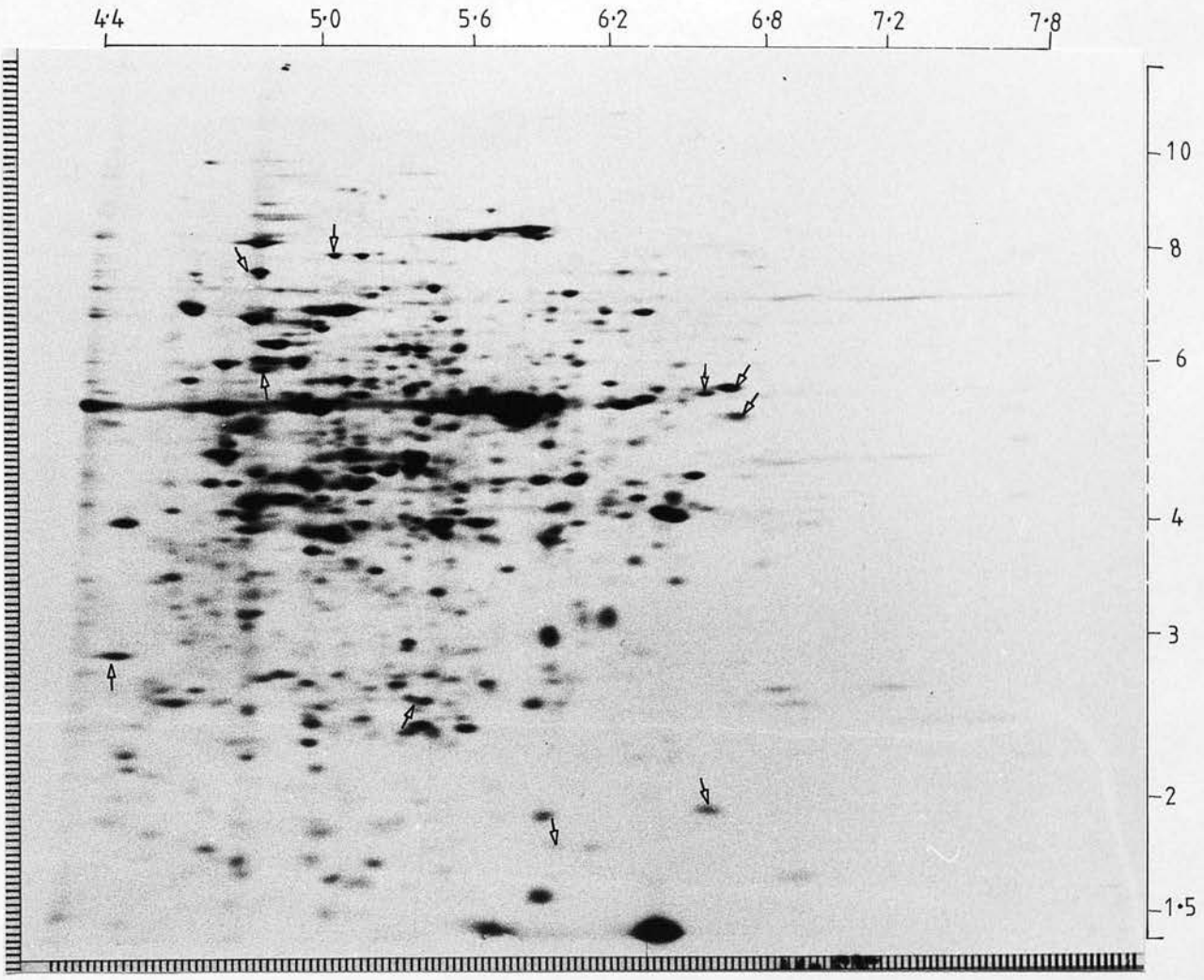
↙ indicates that protein has increased in amount from the day before

↗ indicates that protein has decreased in amount on the next day

FIGURE 57


(A)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5


(B)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
72-b


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

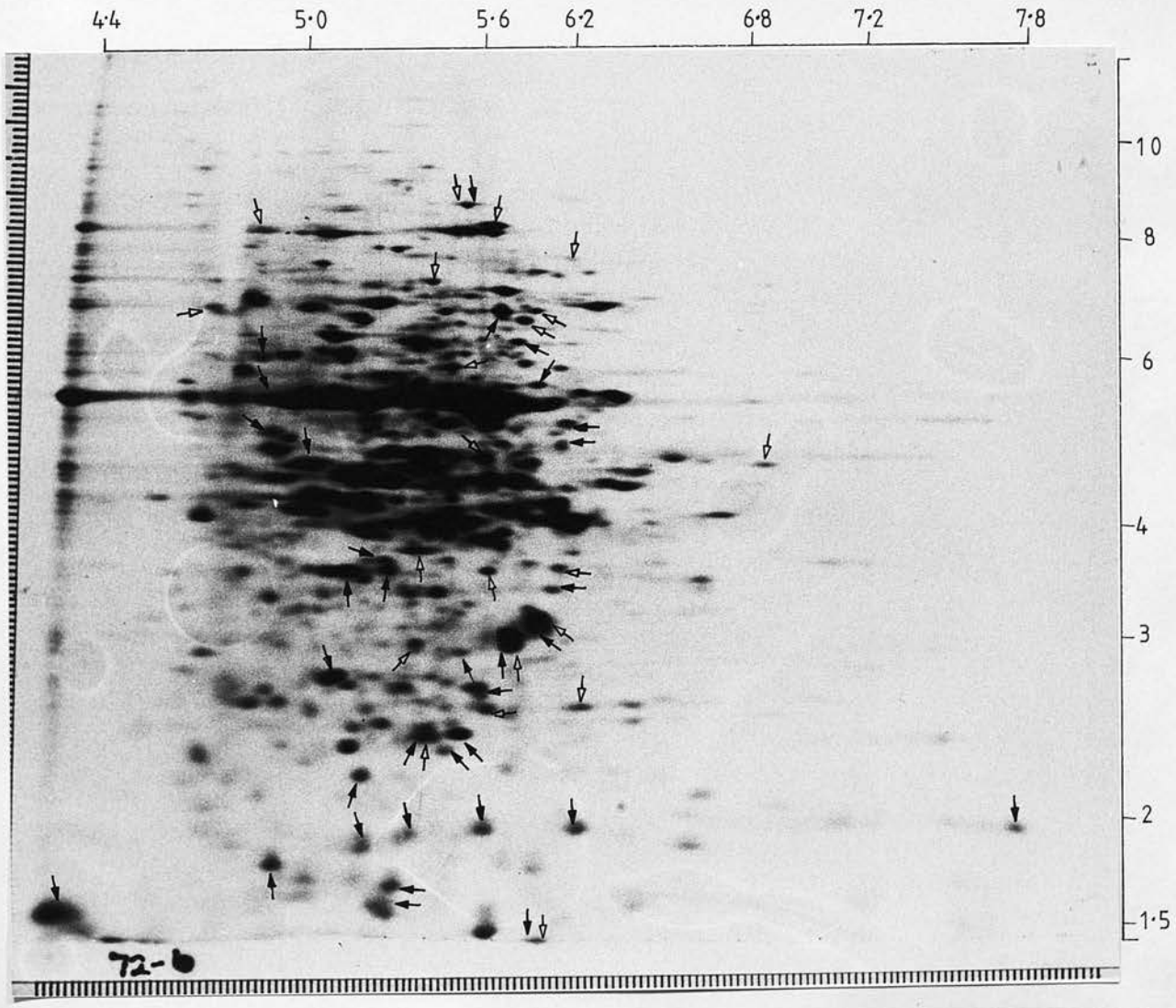
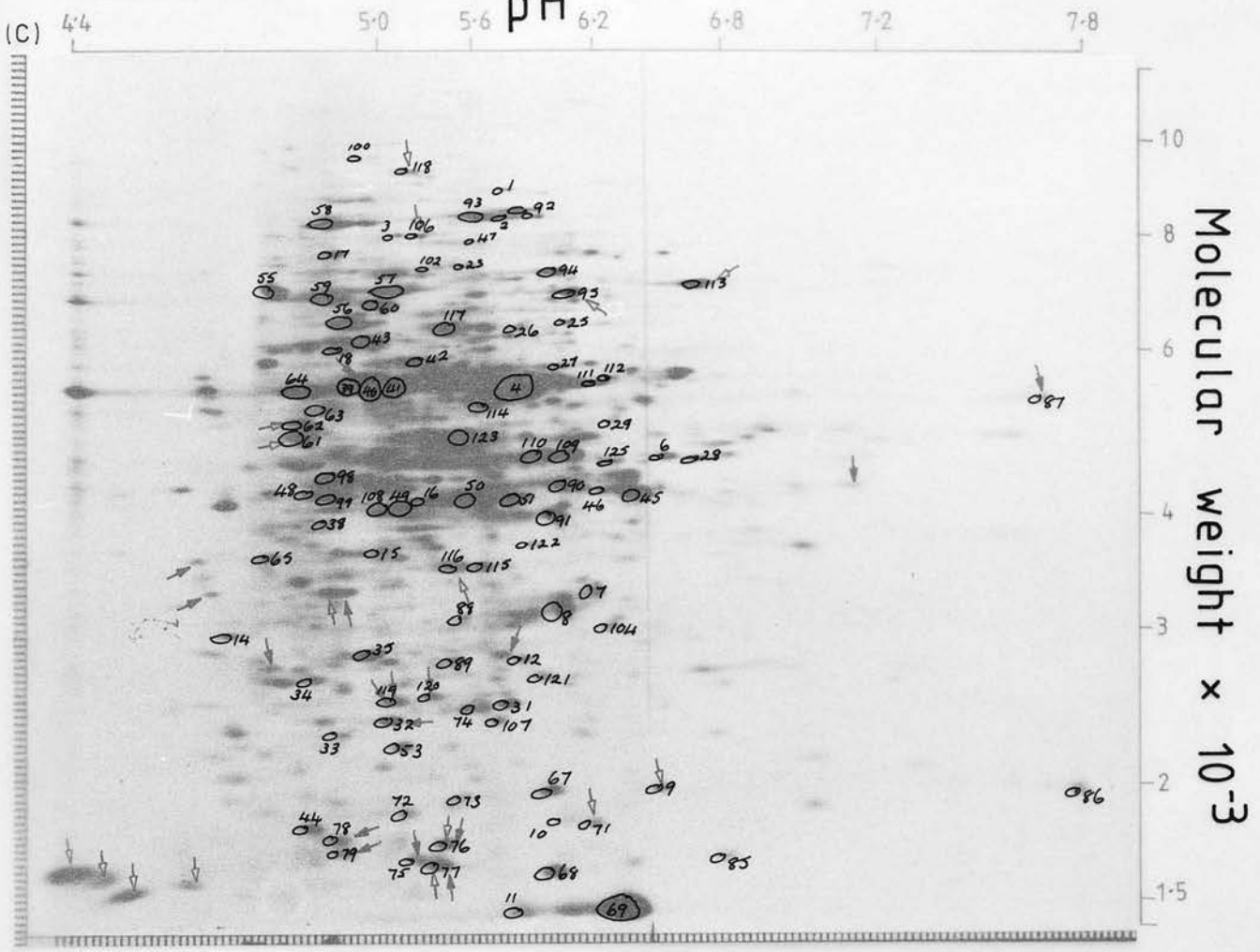
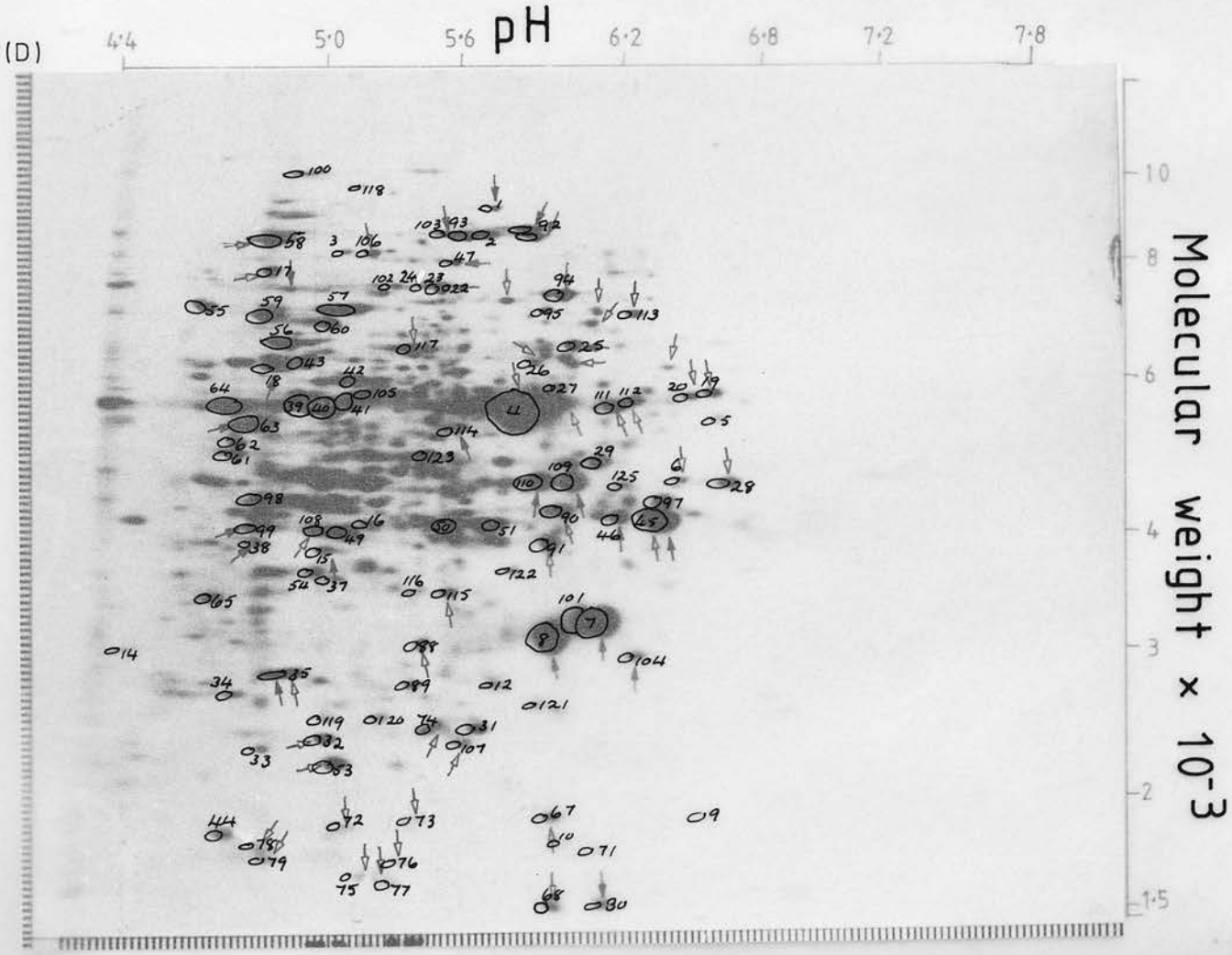
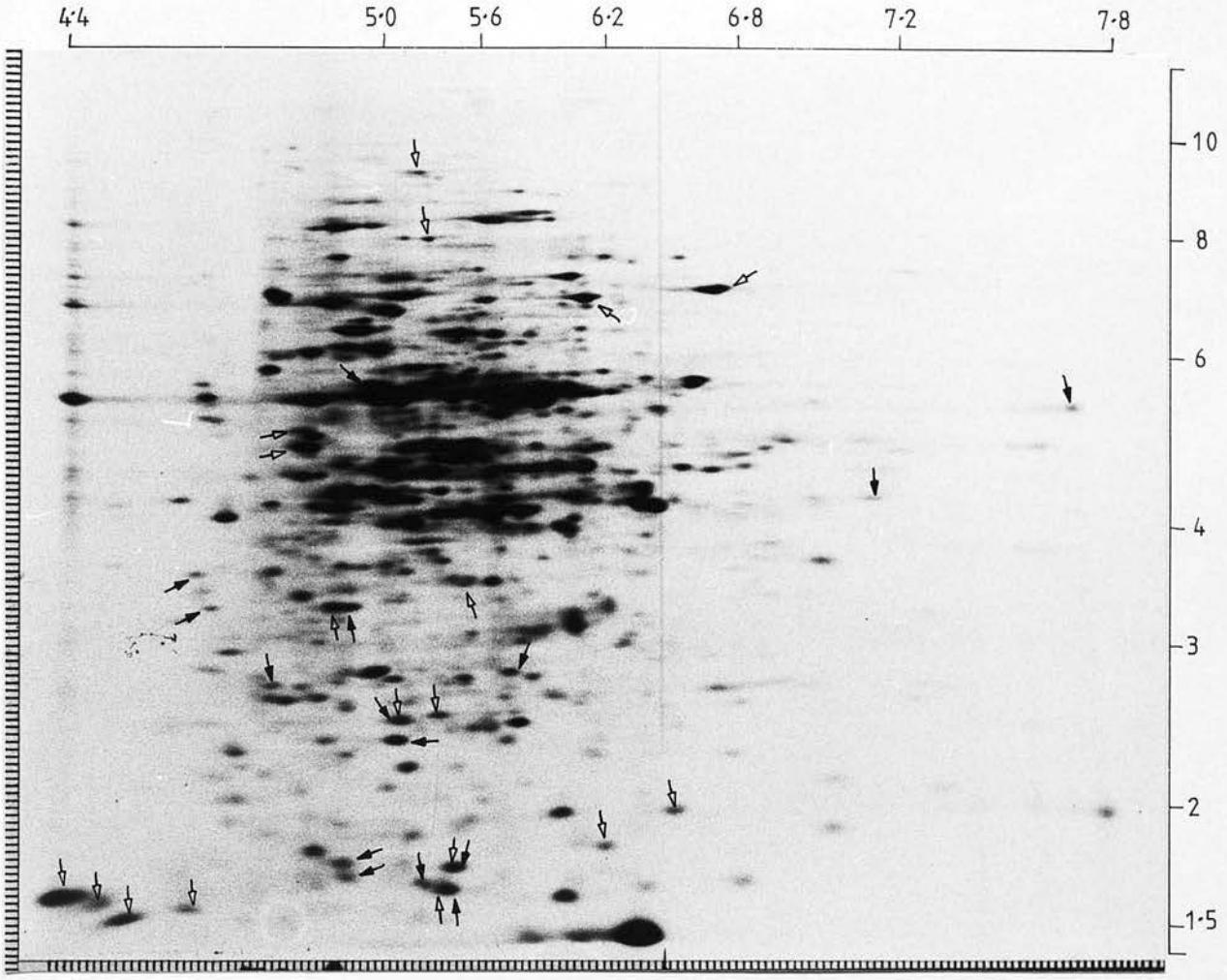

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5
72-b

FIGURE 57


(C)
4·4
5·0
5·6
pH
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5
Molecular weight × 10⁻³


(D)
4·4
5·0
5·6
pH
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5
Molecular weight × 10⁻³


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

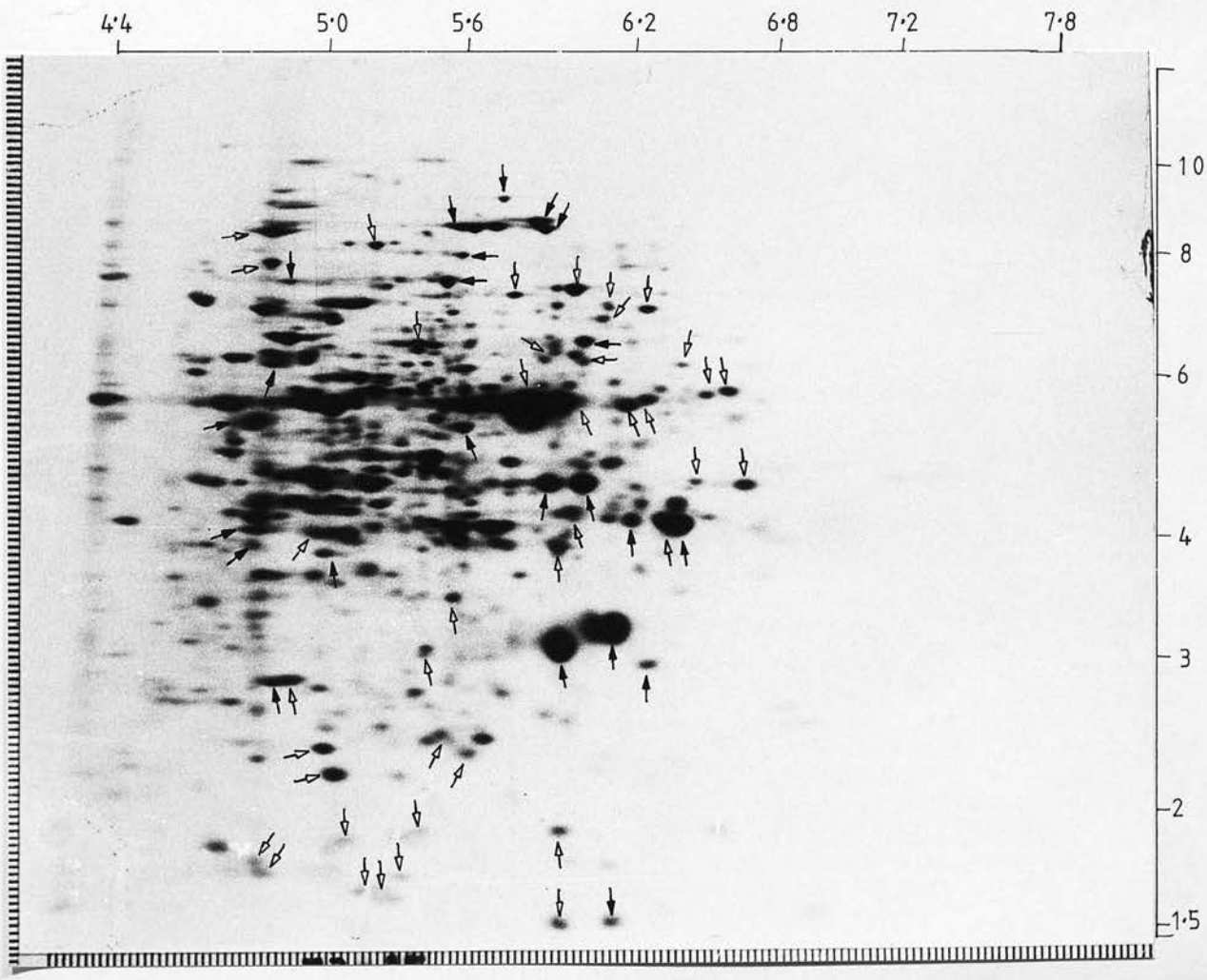
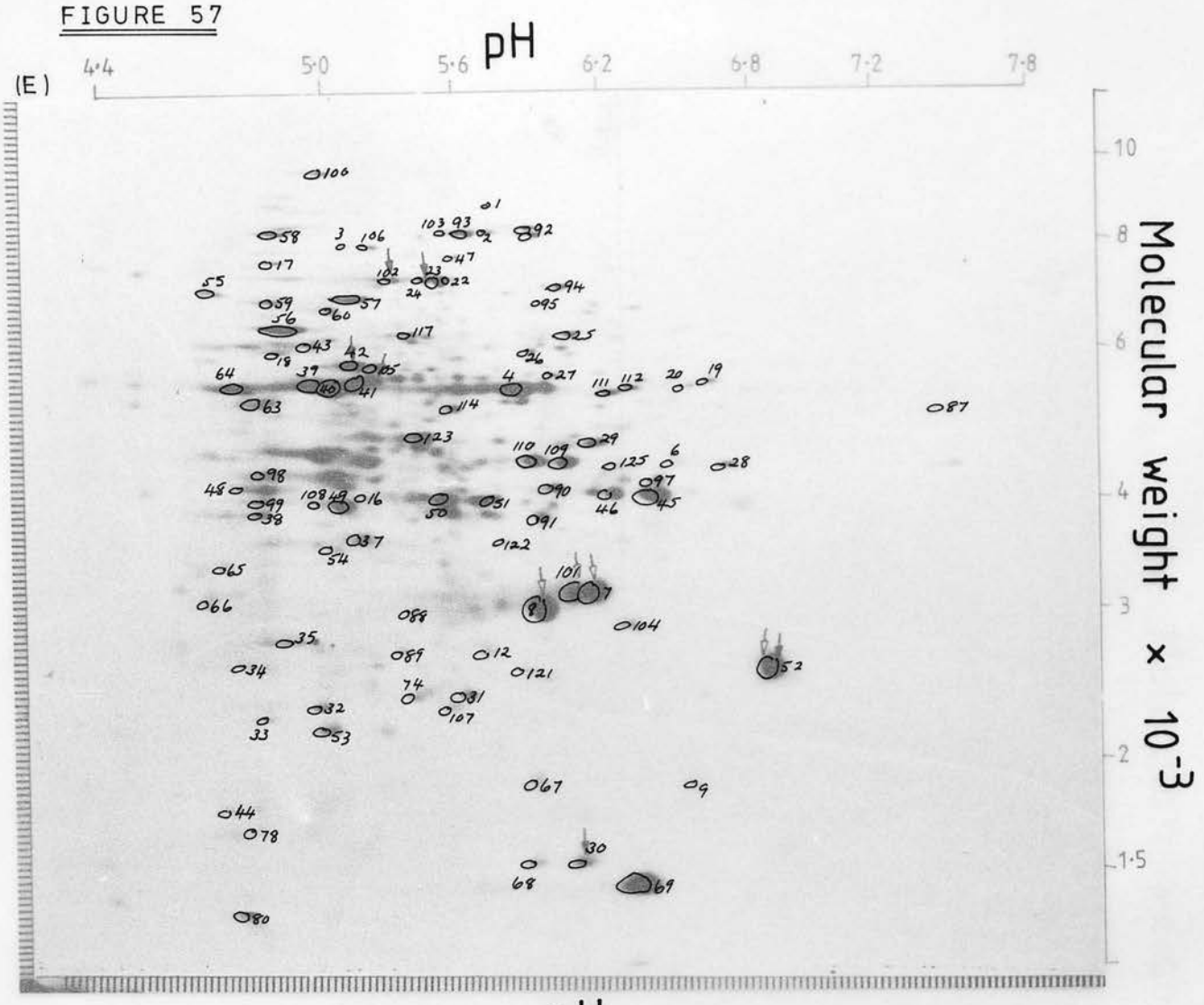
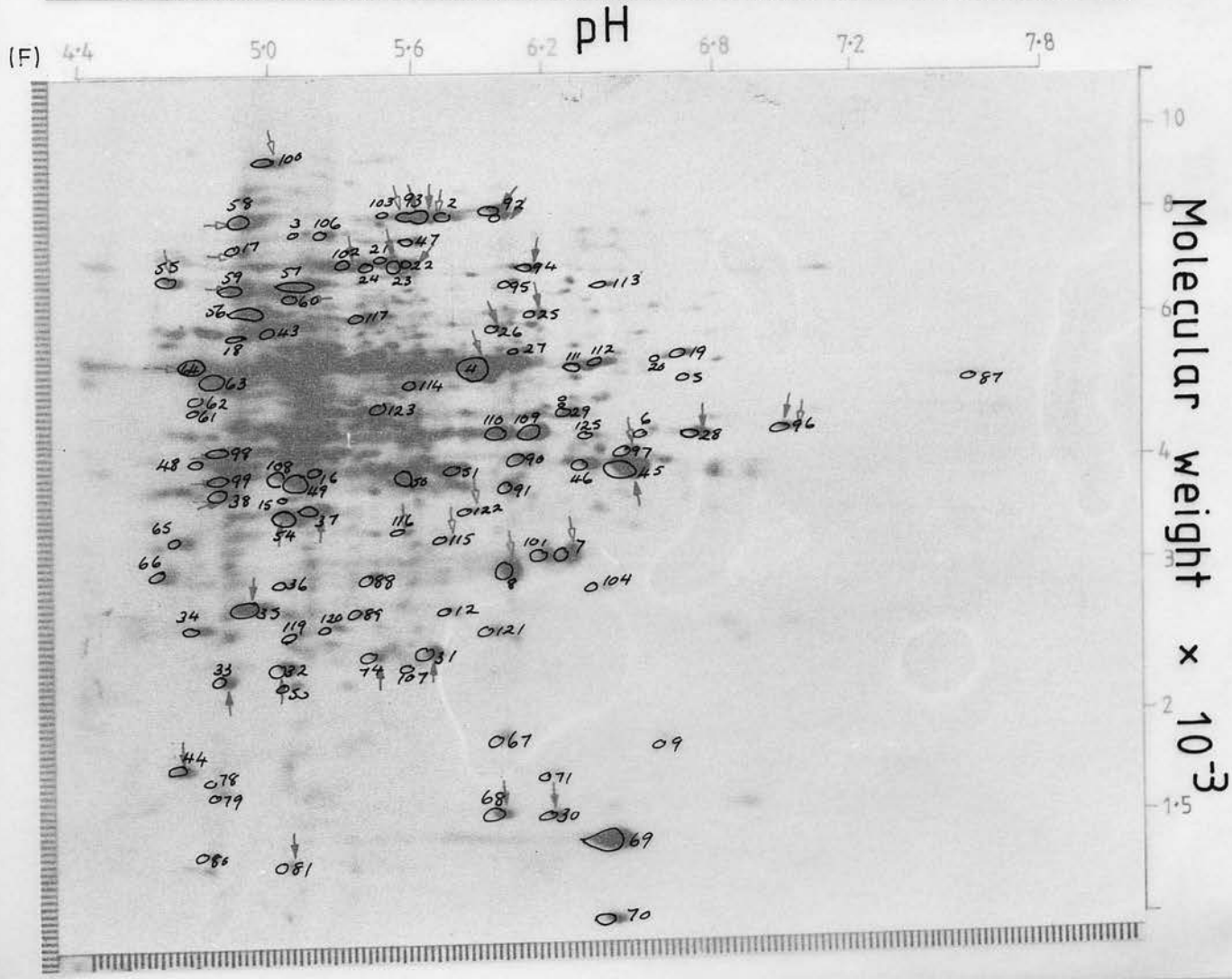
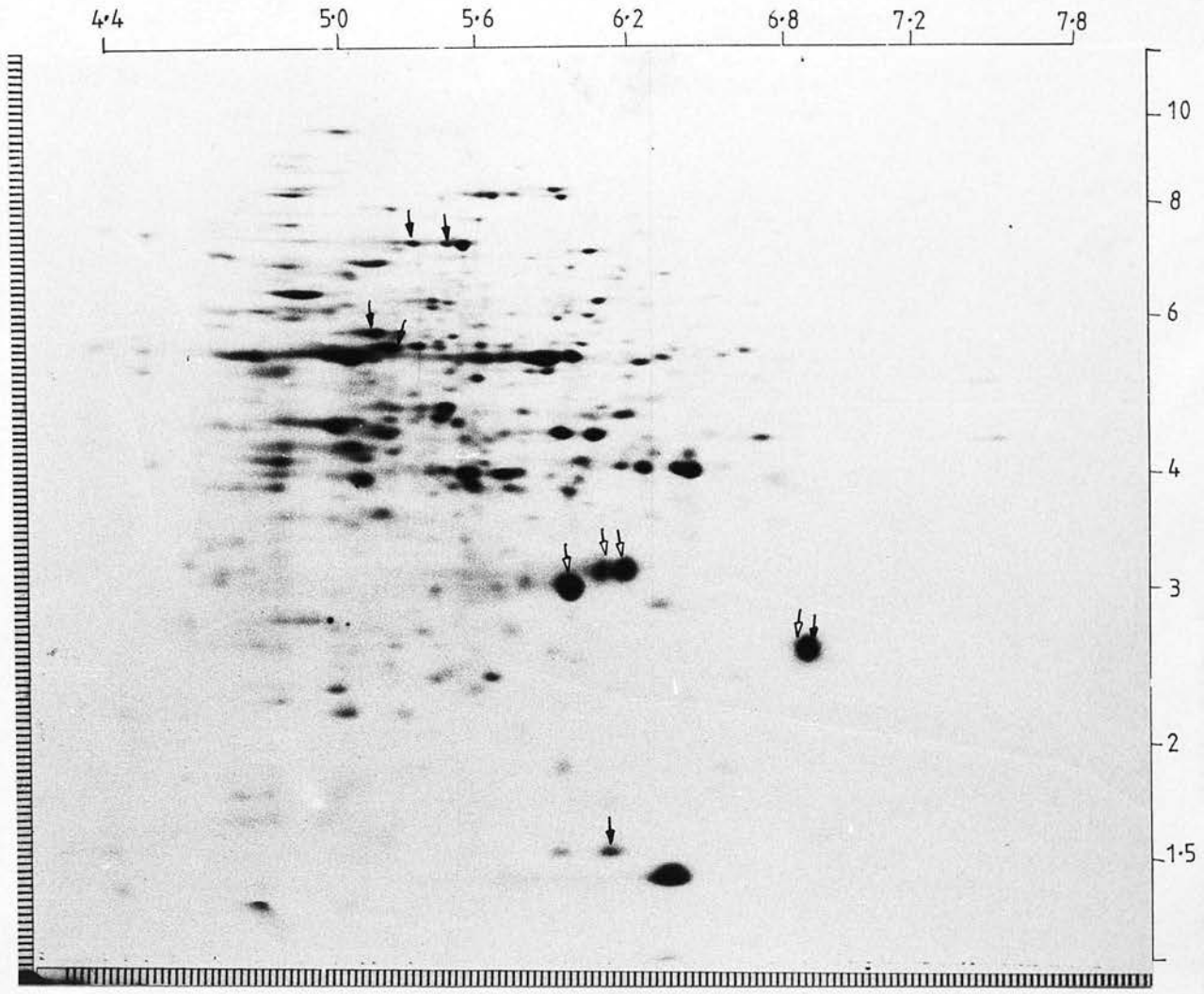

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 57


(E)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5


(F)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

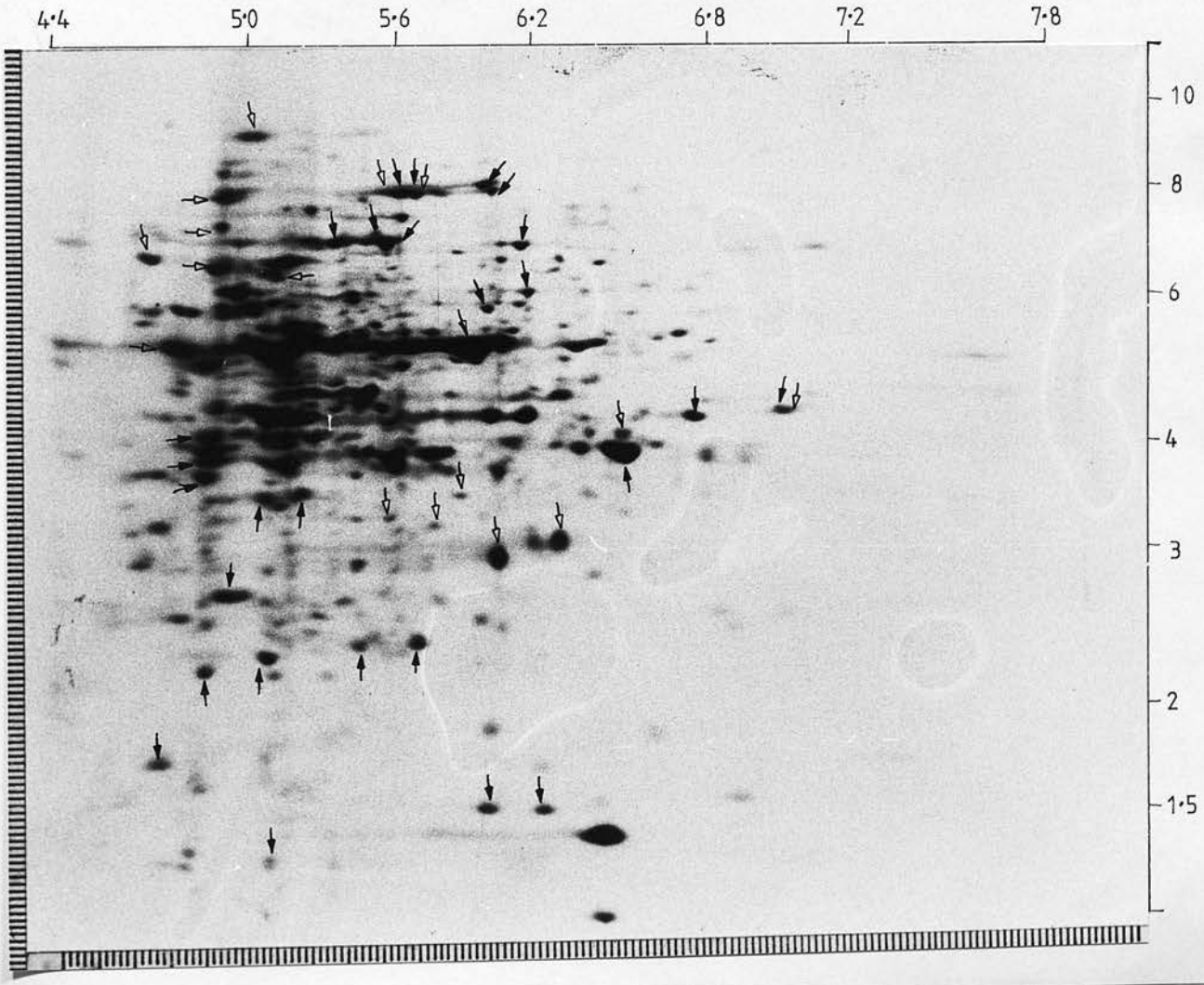
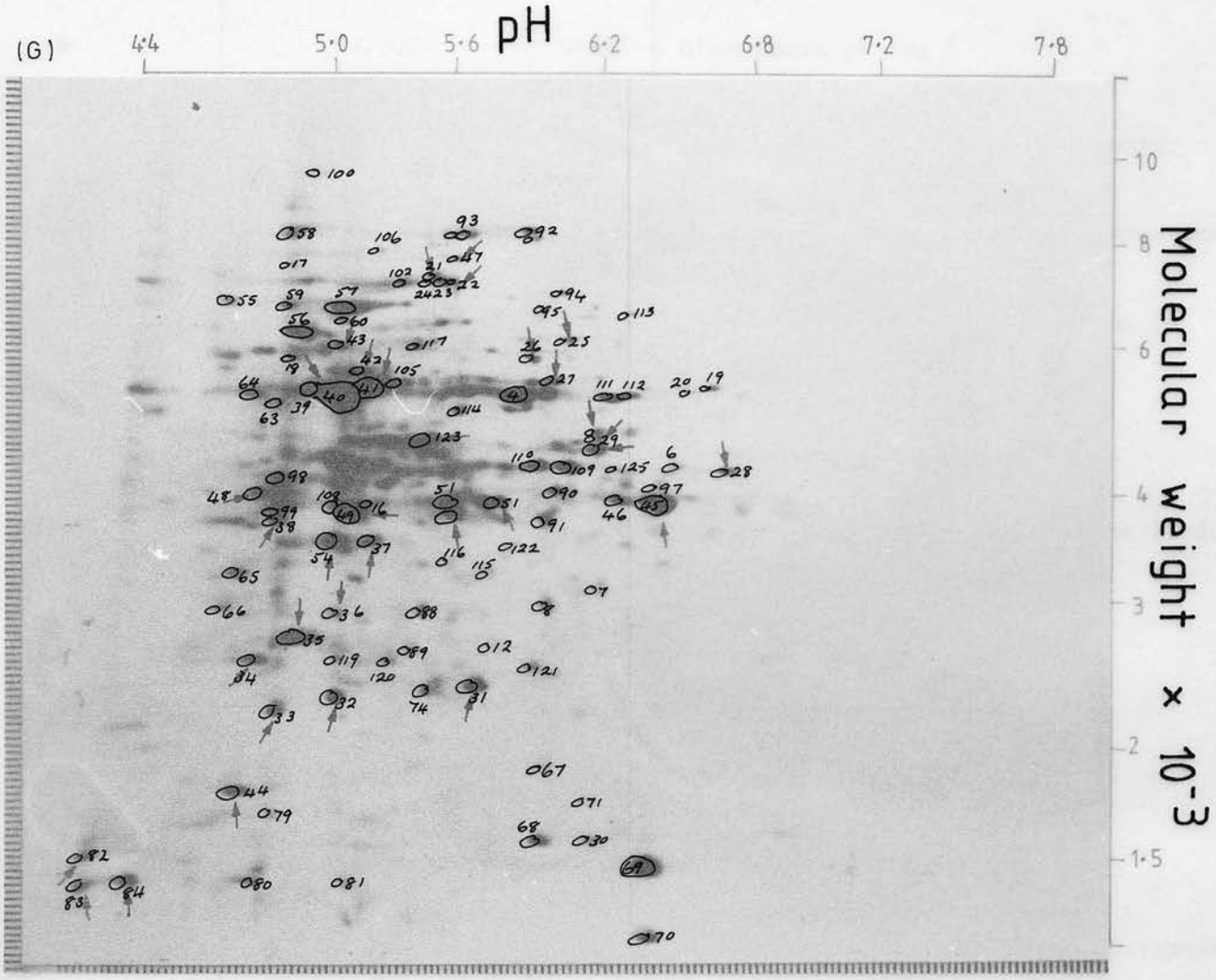

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 57



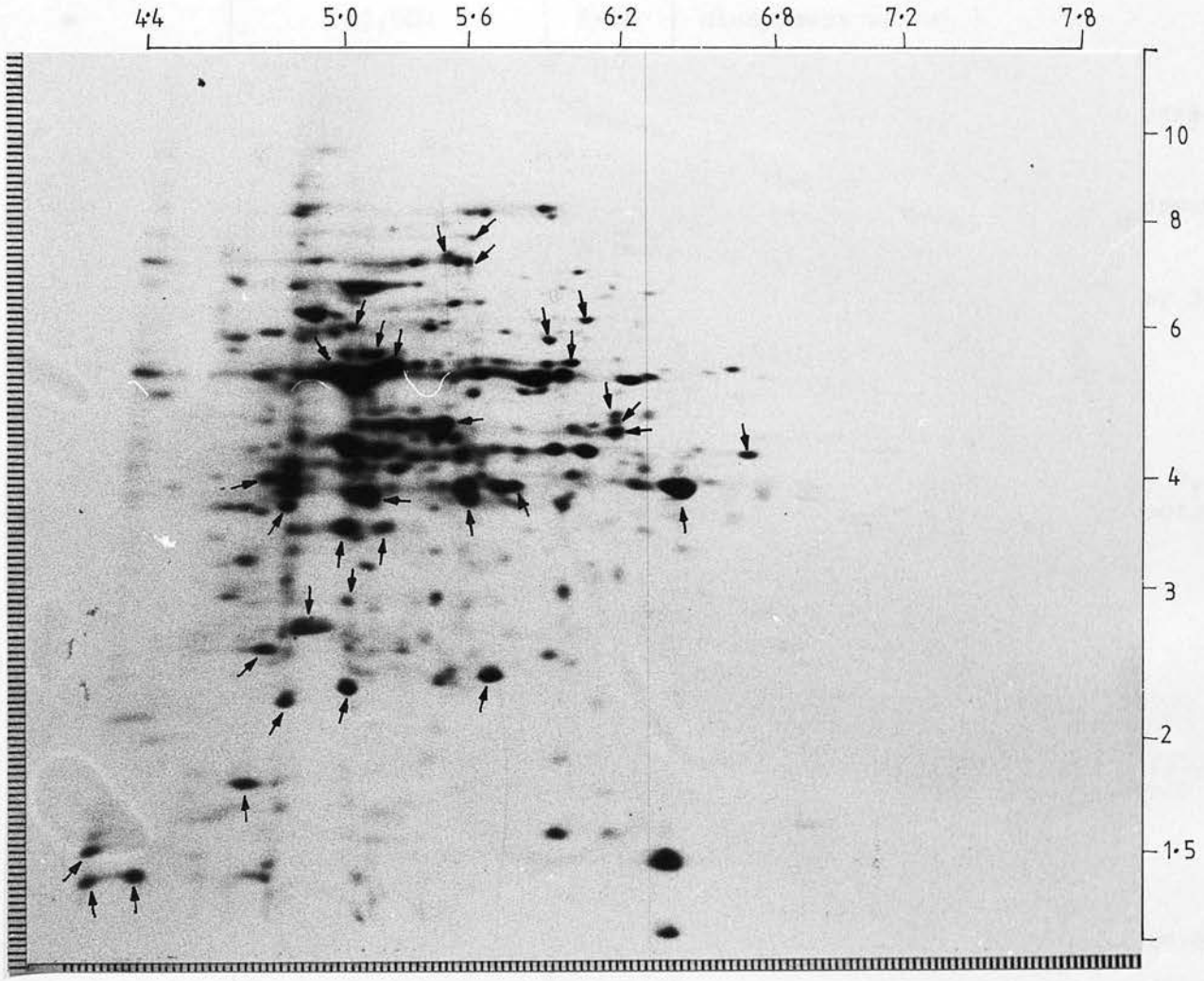

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

Table 8: Catalogue of in vivo labelled proteins in the developing turion of S. polyrrhiza (N)

o overall decrease □ overall increase T transient * no overall change

Protein	Molecular weight (daltons)	pI	Occurrence
● 1	86,000	5·88	decreases after 2 days, disappears at day 5
● 2	82,000	5·68	disappears at day 7
● 3	78,000	5·06	decreases after 1 day, disappears at day 7
o 4	53,500	5·88	decreases throughout turion formation
● 5	51,500	6·77	disappears day 1, reappears day 3, disappears day 4
o 6	44,500	6·58	decreases day 3 & thereafter
o 7	31,500	6·30	increases day 1, decreases day 2, increases day 3, thereafter decreasing
o 8	29,500	6·06	same as protein 7
● 9	19,200	6·74	decreases day 3 & thereafter, disappears day 7
● 10	16,000	6·16	disappears day 4
● 11	14,500	5·91	disappears day 4
o 12	26,500	5·83	increases day 1, thereafter decreasing
● 13	25,000	5·53	decreases day 1, disappears day 2
● 14	28,000	4·57	decreases day 1 & thereafter, disappears day 4
● 15	37,000	5·06	decreases day 1, disappears day 7
o 16	39,000	5·25	decreases day 4 & thereafter
o 17	80,000	4·92	decreases day 1, increases day 3, decreases day 4, increases day 5, decreases day 7

● disappearing ■ appearing

Table 8 continued

	Protein	Molecular weight (daltons)	pI	Occurrence
*	18	78,000	5·00	fluctuates throughout turion formation, but no overall change after 7 days
o	19	56,000	6·74	disappears day 1, reappears day 3, decreasing thereafter
o	20	54,000	6·61	same as protein 19
■	21	72,000	5·53	appears day 5, increases day 7
■	22	70,000	5·61	appears day 3, thereafter increasing
□	23	70,000	5·55	increases day 3 & thereafter
□	24	70,000	5·50	same as protein 23
□	25	62,000	6·13	increases day 1 & thereafter
□	26	59,000	5·95	increases day 4 & thereafter
□	27	55,000	6·06	increases day 1 & thereafter
■	28	44,000	6·80	appears day 1, thereafter increasing
■	29	46,000	6·34	same as protein 28
■	30	15,500	6·33	appears day 1, disappears day 2, appears day 3 increasing thereafter, decreases day 7
□	31	23,400	5·72	increases day 1 & thereafter
□	32	23,000	5·06	same as protein 31
□	33	22,000	4·95	increases day 5 & thereafter
□	34	25,000	4·83	increases day 1 & thereafter
□	35	27,000	5·98	increases day 1, decreases day 4, thereafter increasing

Table 8 continued

Protein		Molecular weight (daltons)	pI	Occurrence
■	36	30,000	5•02	appears day 5 & thereafter increases
■	37	36,500	5•02	appears day 3, increasing thereafter
□	38	37,500	4•92	increases day 3 & thereafter
□	39	52,000	5•02	increases day 1 & thereafter
□	40	52,000	5•06	same as protein 39
□	41	53,000	5•16	same as protein 39
□	42	56,000	5•16	same as protein 39
*	43	62,000	5•06	remains fairly constant
□	44	17,300	4•86	increases day 1, increases day 7
□	45	40,000	6•51	increases day 3 & thereafter
□	46	40,000	6•42	same as protein 45
□	47	76,000	5•56	same as protein 45
□	48	40,000	4•86	increases day 7
□	49	37,500	5•12	increases day 5 & thereafter
□	50	38,500	5•58	increases day 3 & thereafter
■	51	39,000	5•82	appears day 3, thereafter increasing
T	52	25,500	6•95	appears day 4, disappears day 5
●	53	21,500	5•07	increases day 1 & thereafter, decreases day 4, disappears day 7

Table 8 continued

Protein	Molecular weight (daltons)	pI	Occurrence
■ 54	34,000	5•06	appears day 3, thereafter increasing
o 55	67,000	4•92	decreases day 7
* 56	61,000	5•02	remains constant
* 57	67,000	5•06	remains constant
o 58	80,000	4•92	decreases day 7
o 59	73,000	4•91	decreases day 7
o 60	64,000	5•06	decreases day 7
● 61	48,000	4•85	decreases day 4, disappears day 7
● 62	46,500	4•87	same as protein 61
o 63	49,500	4•90	decreases day 7
o 64	52,000	4•85	decreases day 7
* 65	34,000	4•72	remains constant
■ 66	30,000	4•70	appears day 4, thereafter increasing
o 67	19,000	5•90	decreases day 4 & thereafter
* 68	16,000	5•90	remains constant
o 69	14,300	7•75	decreases day 7
* 70	11,000	7•75	run off gel until day 5
* 71	17,200	6•20	remains constant

Table 8 continued

	Protein	Molecular weight (daltons)	pI	Occurrence
●	72	18,200	5•07	disappears day 4
●	73	18,300	5•46	same as protein 72
○	74	23,200	5•53	decreases day 2 & thereafter
●	75	16,000	5•15	decreases day 3, disappears day 4
●	76	16,800	5•40	increases day 1 & thereafter, decreases day 3, disappears day 4
●	77	15,800	5•31	same as protein 76
●	78	16,900	4•95	decreases day 5, disappears day 7
○	79	16,300	4•95	decreases day 7
*	80	14,500	4•83	run off gel until day 4, no apparent change
*	81	14,200	5•10	run off gel until day 5, no apparent change
■	82	15,500	4•44	appears day 7
■	83	14,200	4•44	appears day 7
■	84	14,500	4•56	appears day 7
●	85	16,000	6•96	disappears day 2
T	86	19,200	7•80	appears day 1, disappears day 3
●	87	48,000	7•72	disappears day 3, reappears day 4, disappears day 7
*	88	28,600	5•53	remains constant
○	89	26,500	5•47	decreases day 7

Table 8 continued

	Protein	Molecular weight (daltons)	pI	Occurrence
*	90	41,000	6•10	remains constant
*	91	37,800	6•04	remains constant
o	92	83,000	5•80	fluctuates, decreases day 7
o	93	82,000	5•57	same as protein 92
o	94	70,000	6•10	decreases day 7
o	95	67,000	5•95	decreases day 7
T	96	45,000	7•03	appears day 5, disappears day 7
o	97	42,000	6•51	decreases day 7
*	98	41,500	4•92	remains fairly constant
*	99	39,000	4•92	remains fairly constant
□	100	91,000	5•02	increases day 3 & thereafter, decreases day 7
●	101	31,000	6•18	increases day 3, decreases day 5 disappears day 7
□	102	70,000	5•30	increases day 3 & thereafter
●	103	82,000	5•55	increases day 3 & thereafter, disappears day 7
●	104	29,000	6•41	increases day 2 & thereafter, disappears day 7
*	105	54,000	5•25	increases throughout turion formation
o	106	78,000	5•06	decreases day 7
●	107	23,000	5•62	decreases day 4 & thereafter, disappears day 7

Table 8 continued

	Protein	Molecular weight (daltons)	pI	Occurrence
*	108	37,500	5•03	remains constant
*	109	42,000	6•13	remains constant
*	110	42,000	5•96	remains constant
*	111	53,000	6•35	remains constant
*	112	53,500	6•41	remains constant
o	113	67,000	6•41	decreases day 3 & thereafter
*	114	49,500	5•62	remains constant
o	115	35,000	5•58	decreases day 4 & thereafter
o	116	35,500	5•53	decreases day 5 & thereafter
*	117	61,000	5•40	remains fairly constant
●	118	91,000	5•63	disappears day 4
o	119	23,800	5•07	decreases day 3 & thereafter
*	120	24,000	5•79	remains fairly constant
o	121	25,000	5•90	decreases day 2 & thereafter
o	122	35,000	5•87	decreases day 4 & thereafter
*	123	46,000	5•46	remains constant
*	125	42,500	6•22	remains constant
*	126	38,000	5•60	remains fairly constant

Figure 58

The pattern of *in vivo* labelled proteins during turion formation in the mother fronds associated with the turions of *S. polyrrhiza* (N). Tissue was pulse labelled for 3 hours with ^{35}S methionine and soluble protein was extracted and loaded onto 2-dimensional polyacrylamide gels. Equal amounts of soluble protein were applied to each gel (100 µg). Labelled proteins were visualised by autoradiography.

a) day 0 (untreated) 101,000 cpm

b) day 1 90,000 cpm

c) day 2 41,000 cpm

d) day 3 41,000 cpm

e) day 4 39,000 cpm

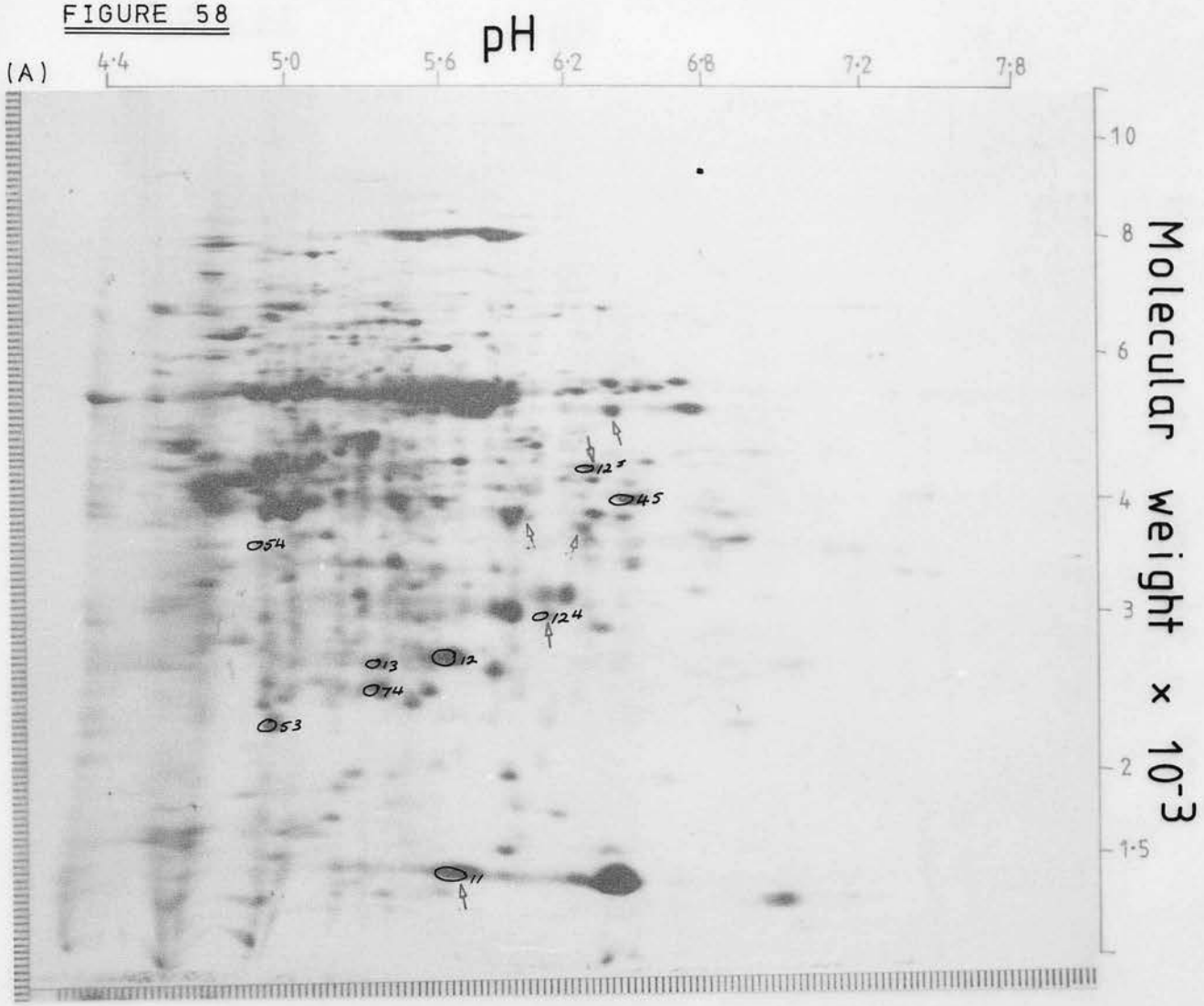
f) day 5 31,000 cpm

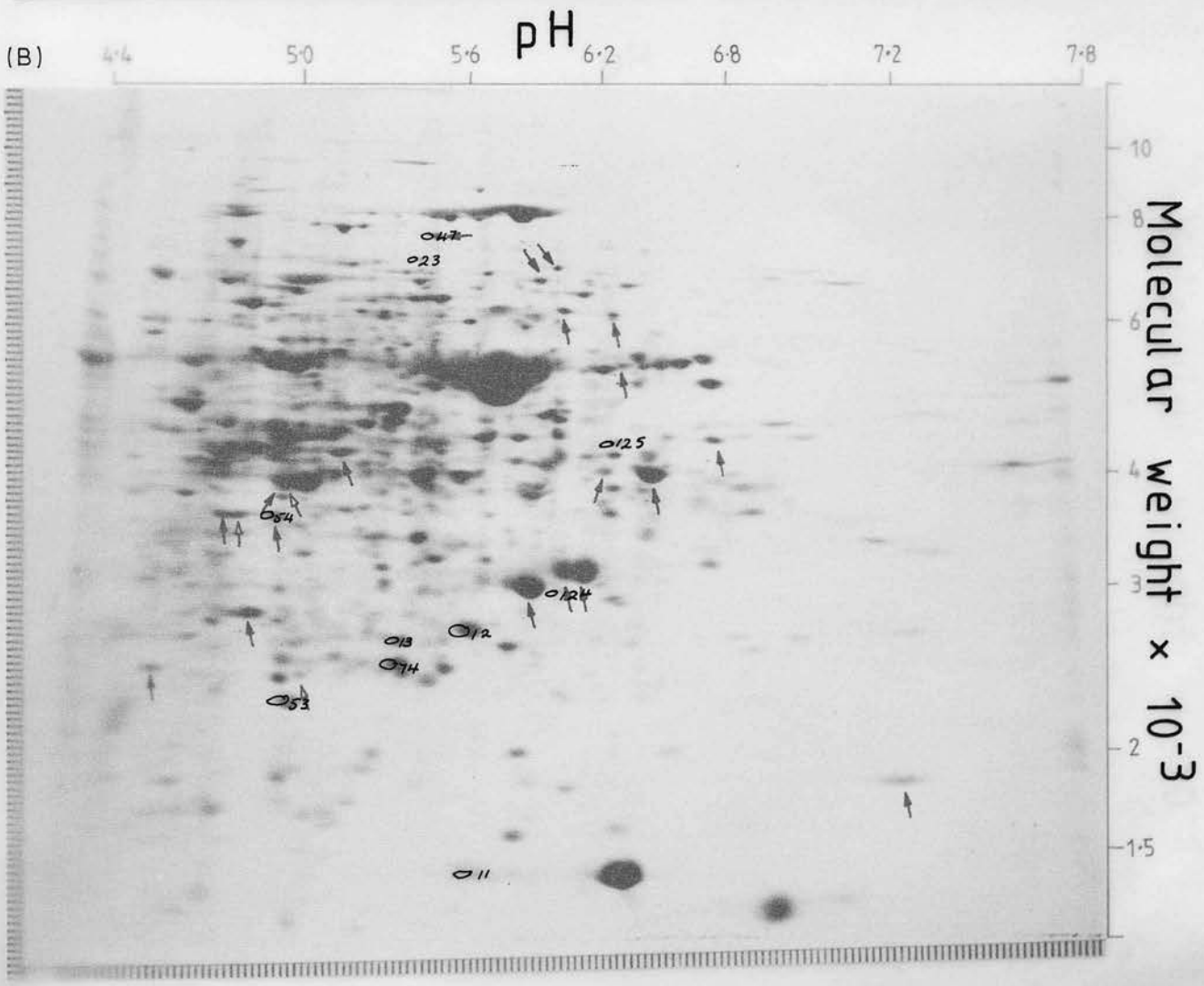
g) day 7 33,000 cpm

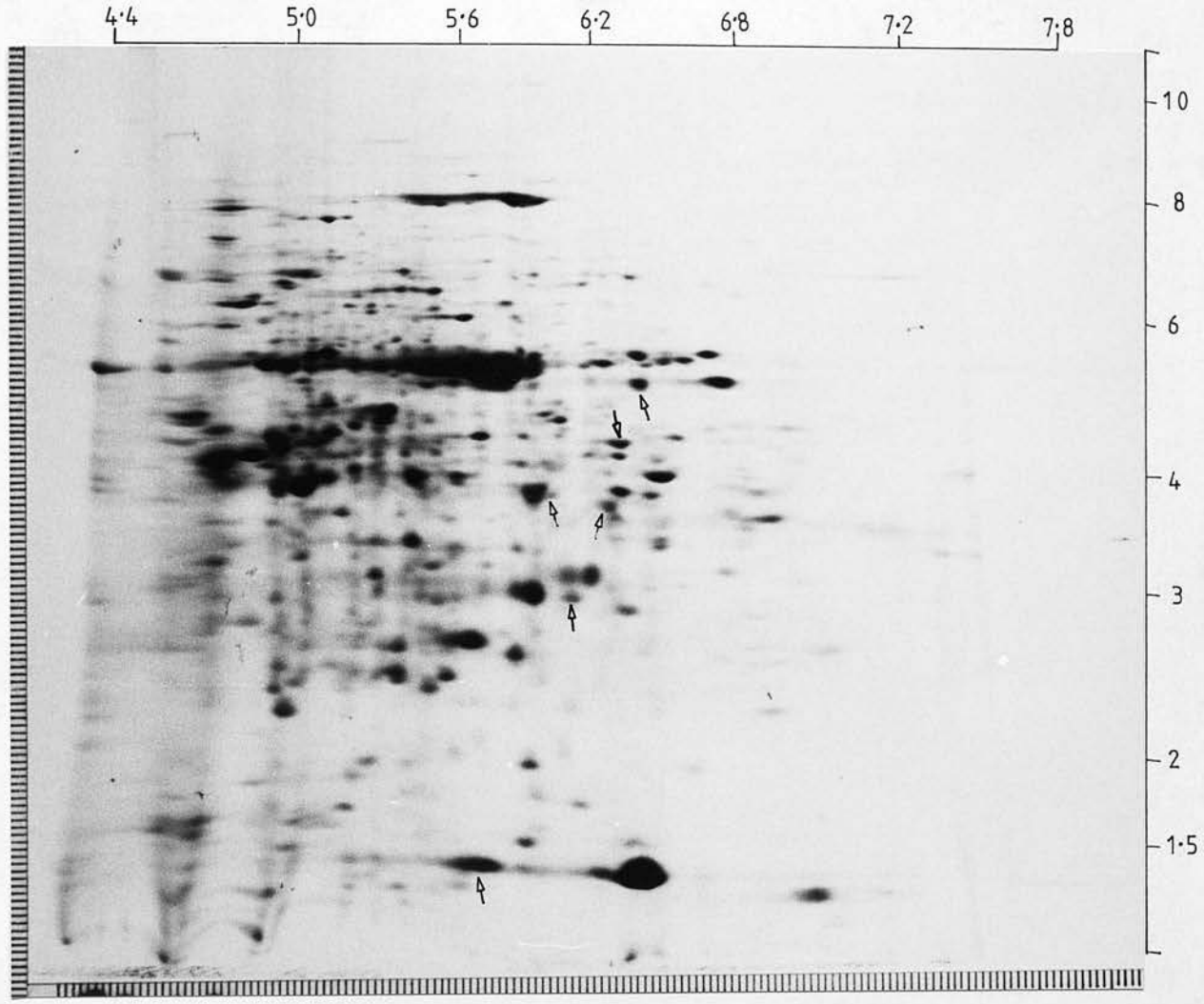
↙ indicates that the protein has increased in amount from the day before

↙ indicates that the protein has decreased in amount on the next day

FIGURE 58


(A)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10-3
10
8
6
4
3
2
1·5
012
045
054
0124
013
012
074
053
011


(B)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10-3
10
8
6
4
3
2
1·5
047
023
0125
054
0124
012
013
074
053
011


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

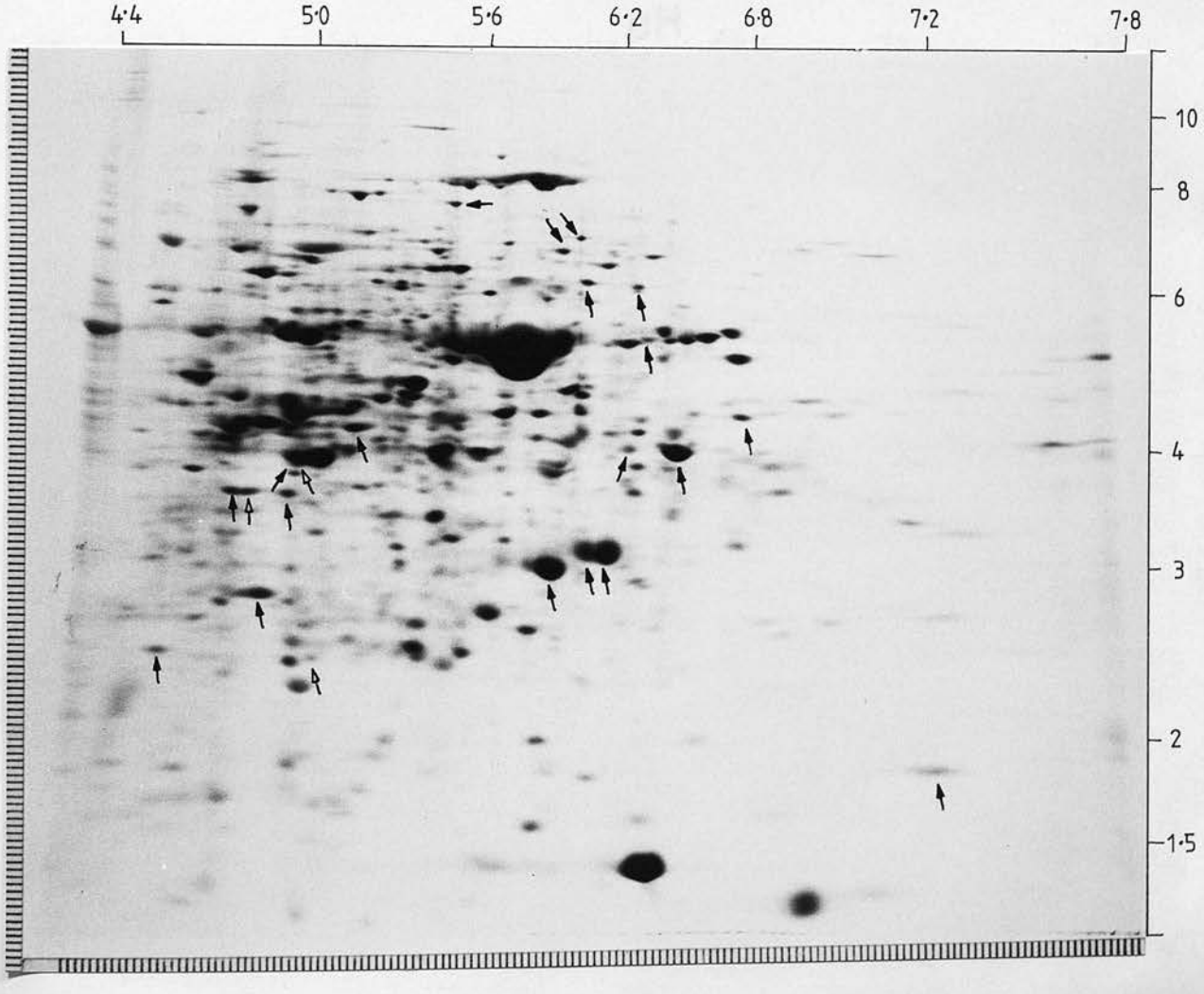
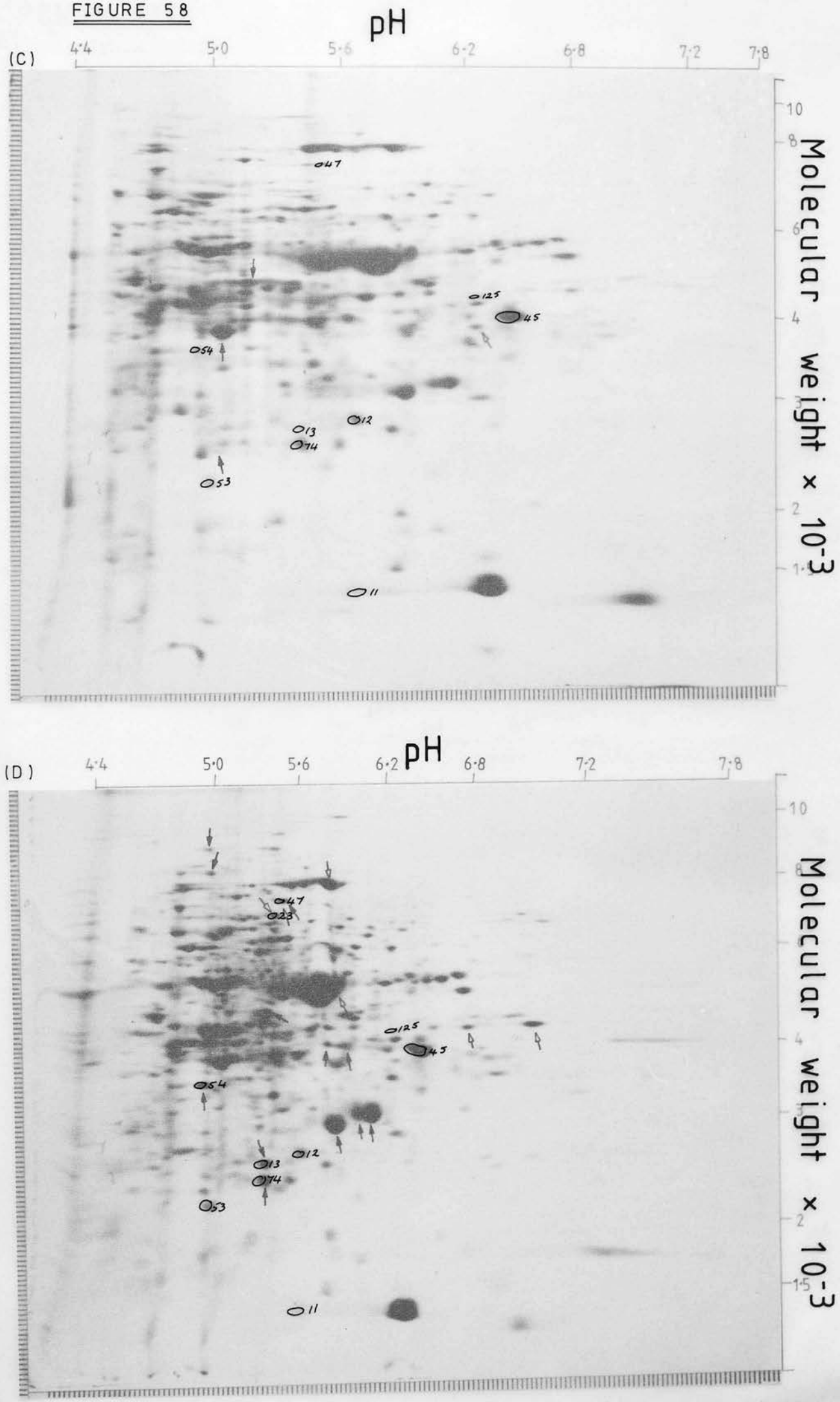
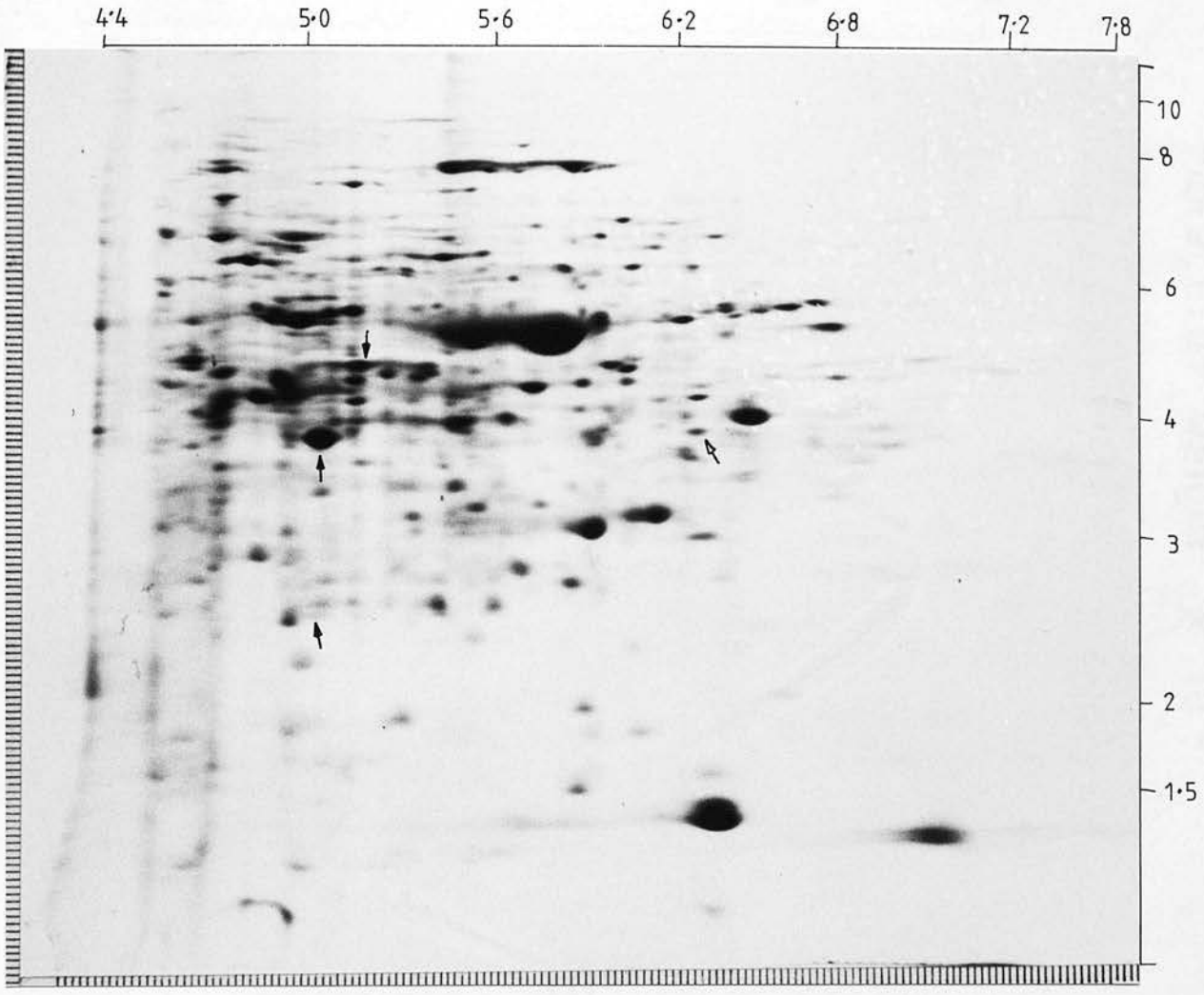

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 58


(C)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10-3
10
8
6
4
3
2
1·5
047
0125
045
054
012
013
074
053
011
(D)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10-3
10
8
4
3
2
1·5
047
023
0125
043
054
012
013
074
053
011


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

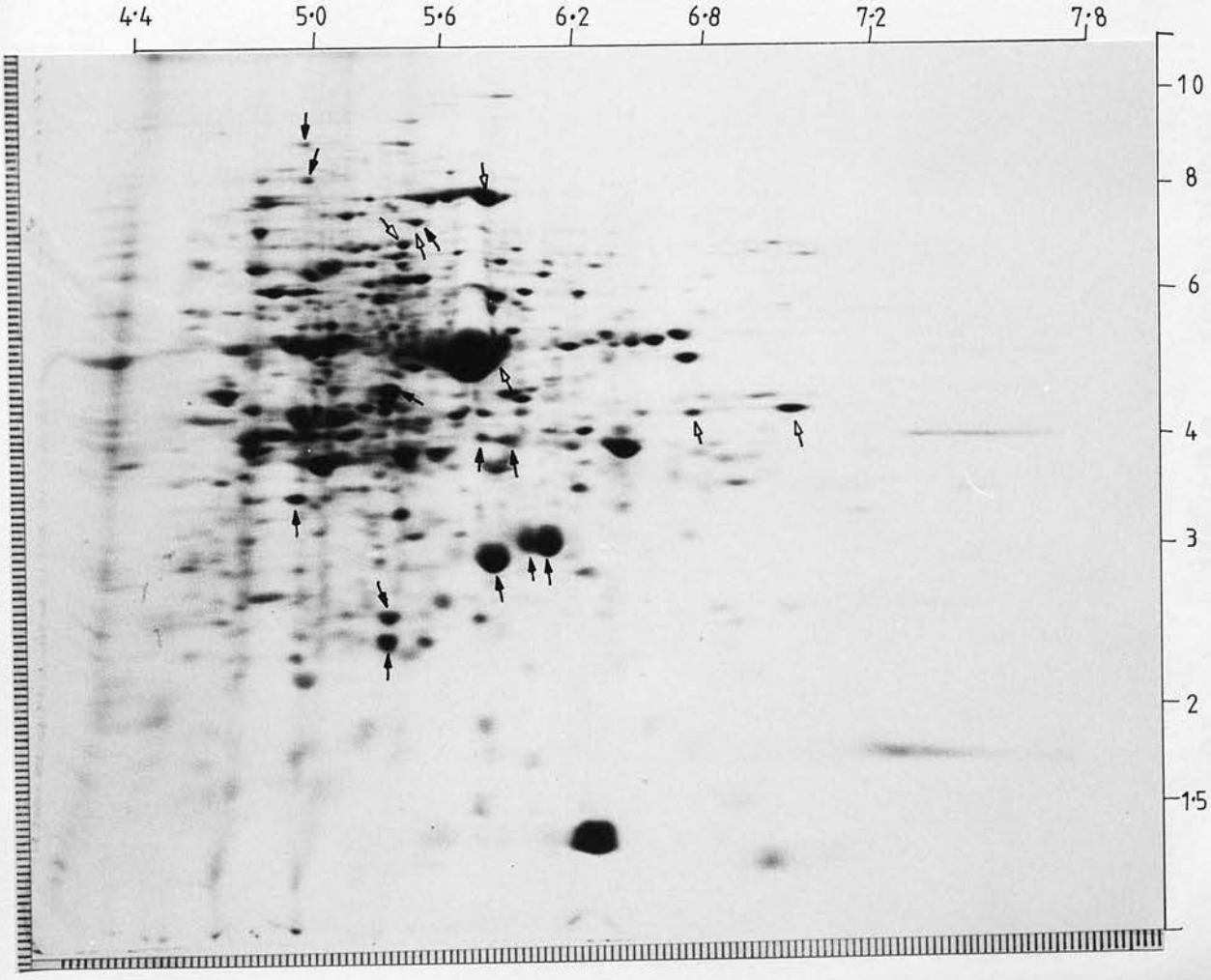
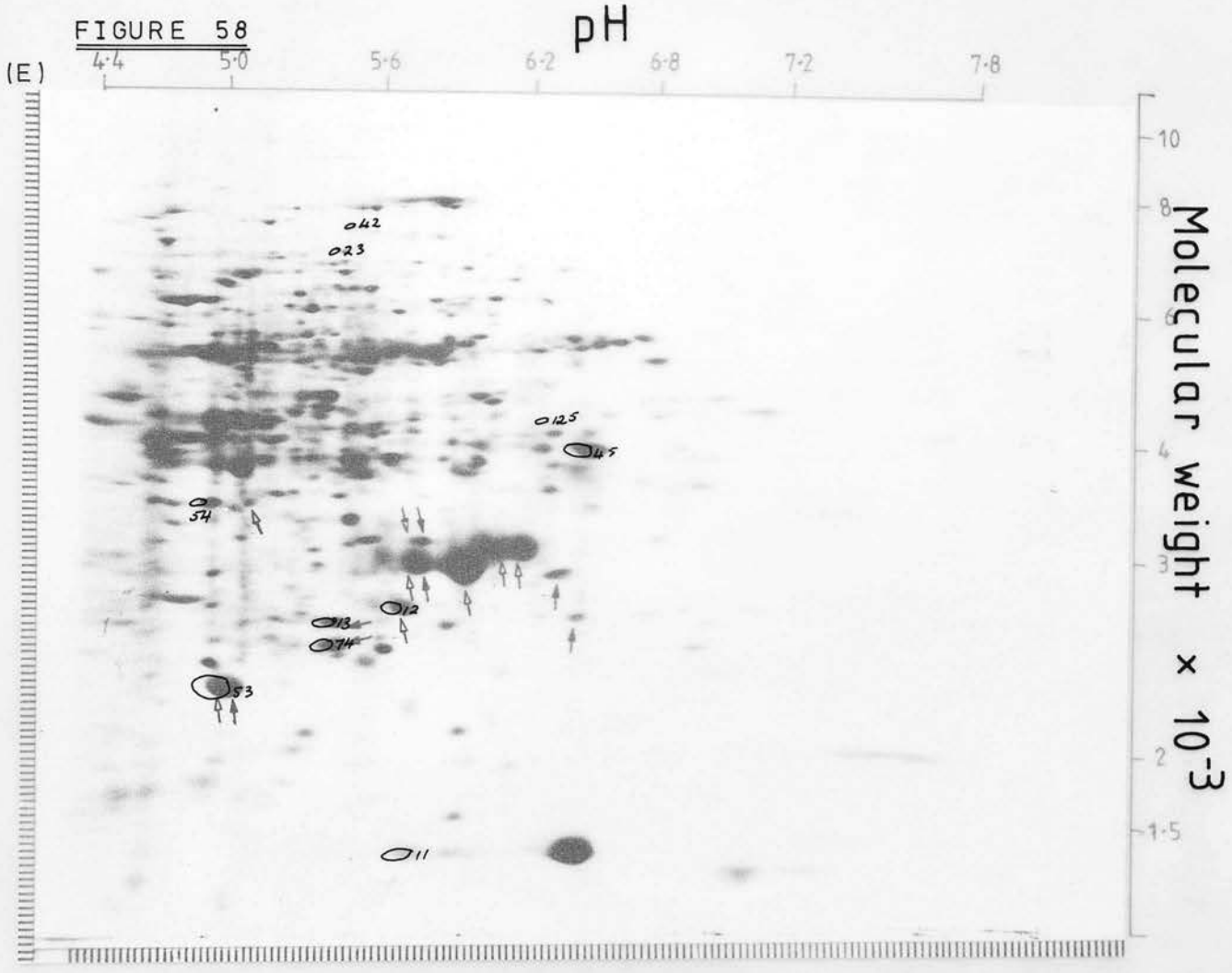
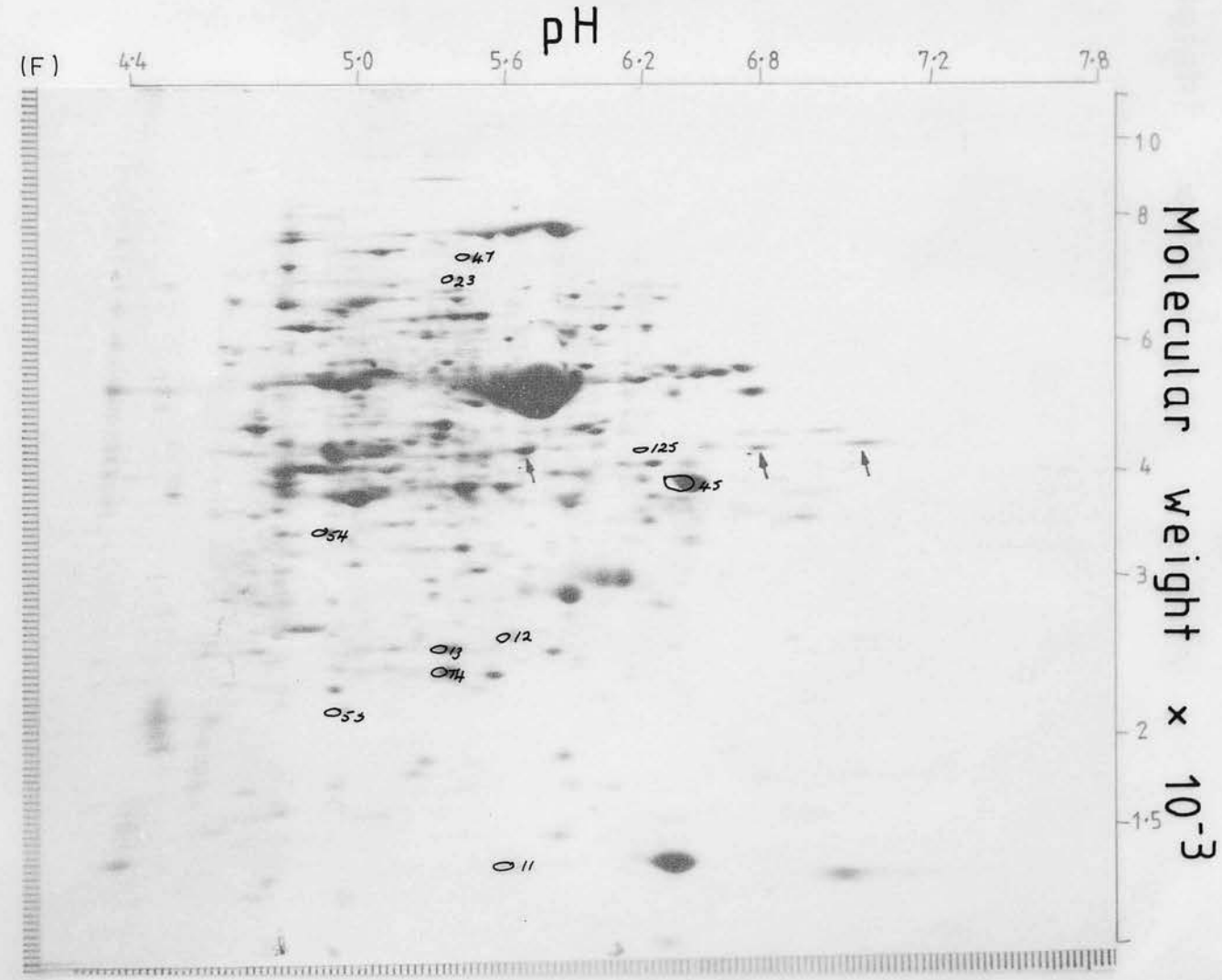
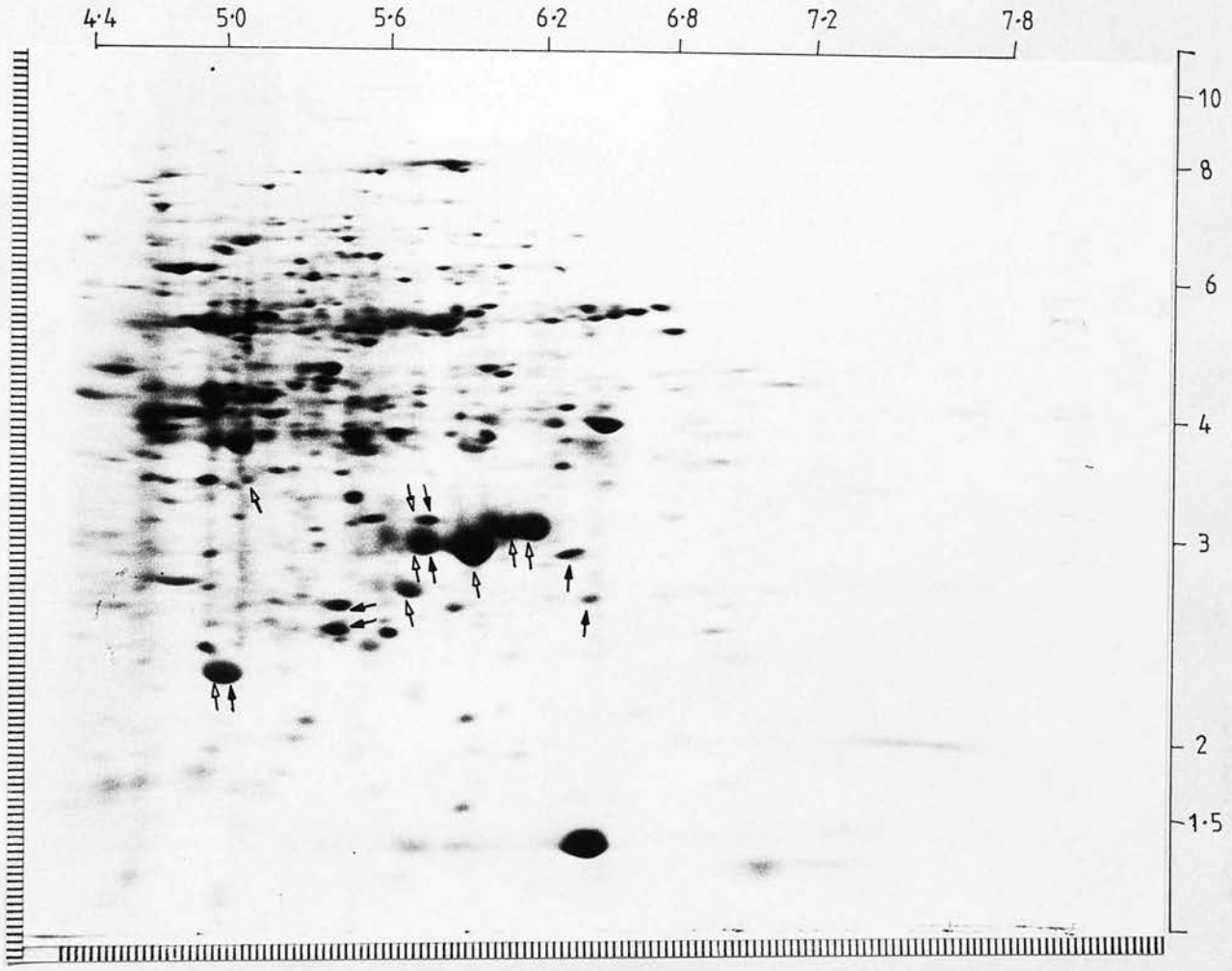

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 58


(E)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
042
023
0125
045
054
012
013
074
053
011


(F)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
047
023
0125
045
054
012
013
074
053
011


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

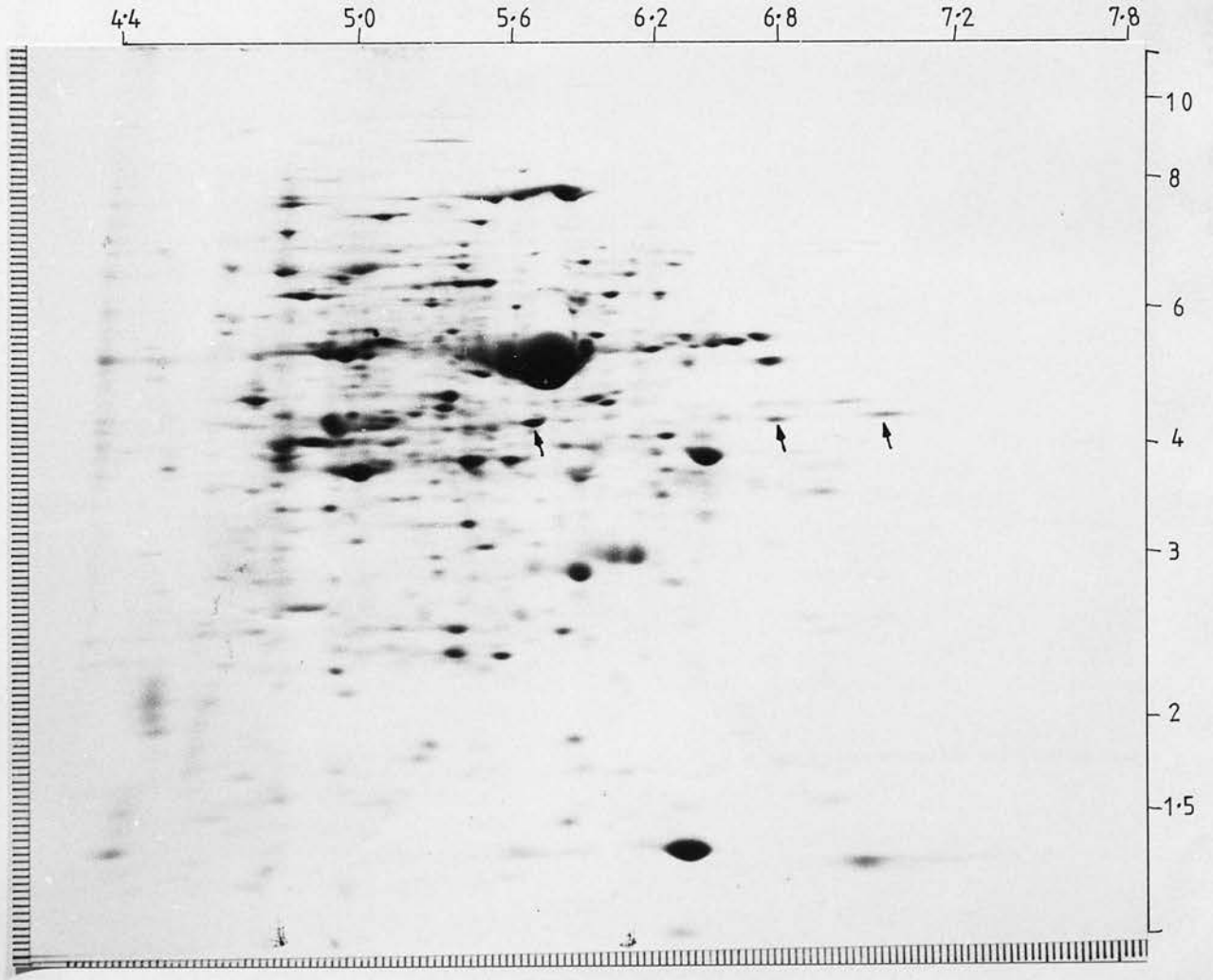
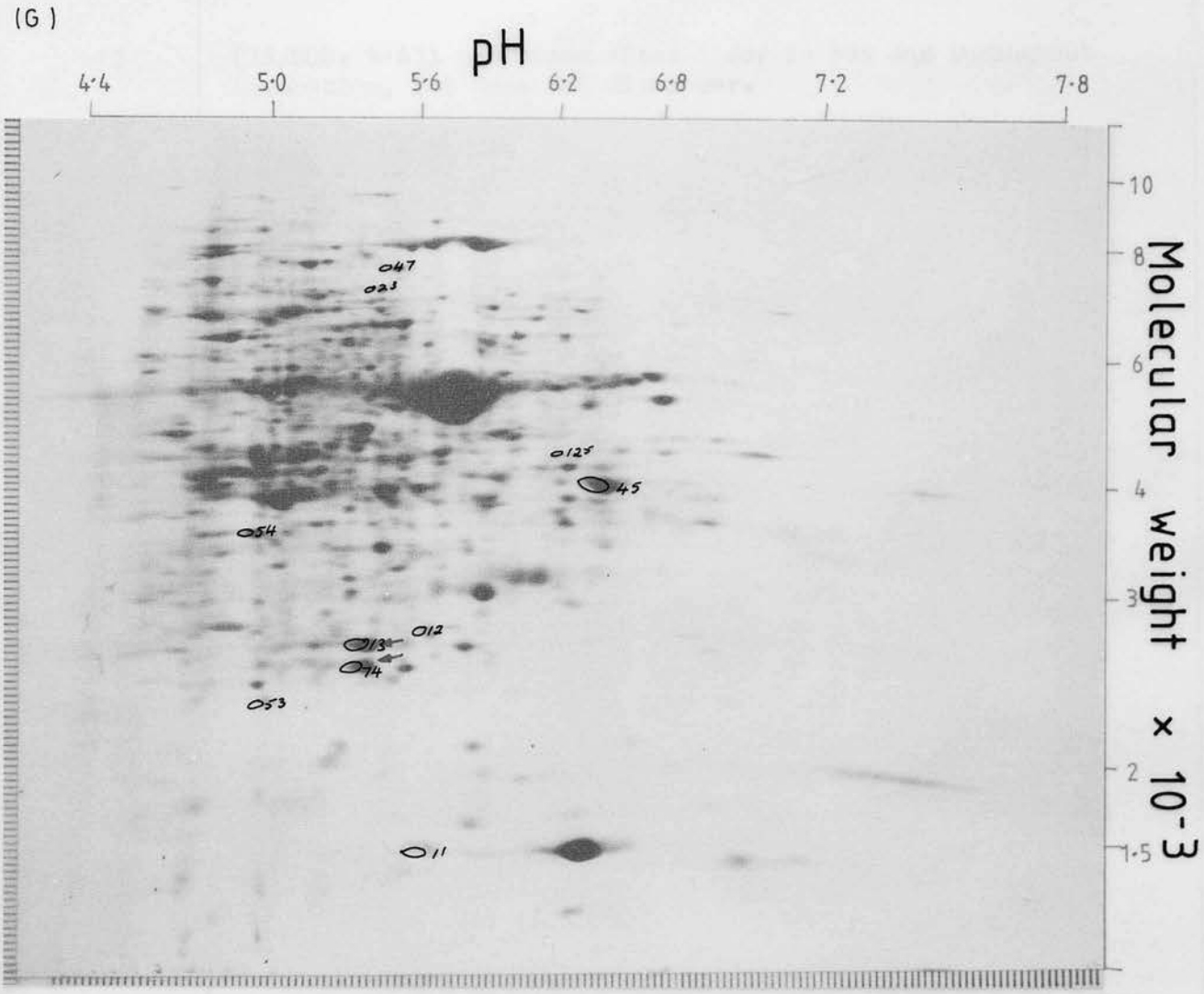

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 58


(G)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10-3
10
8
6
4
3
2
1·5
047
023
0125
045
054
012
013
074
053
011

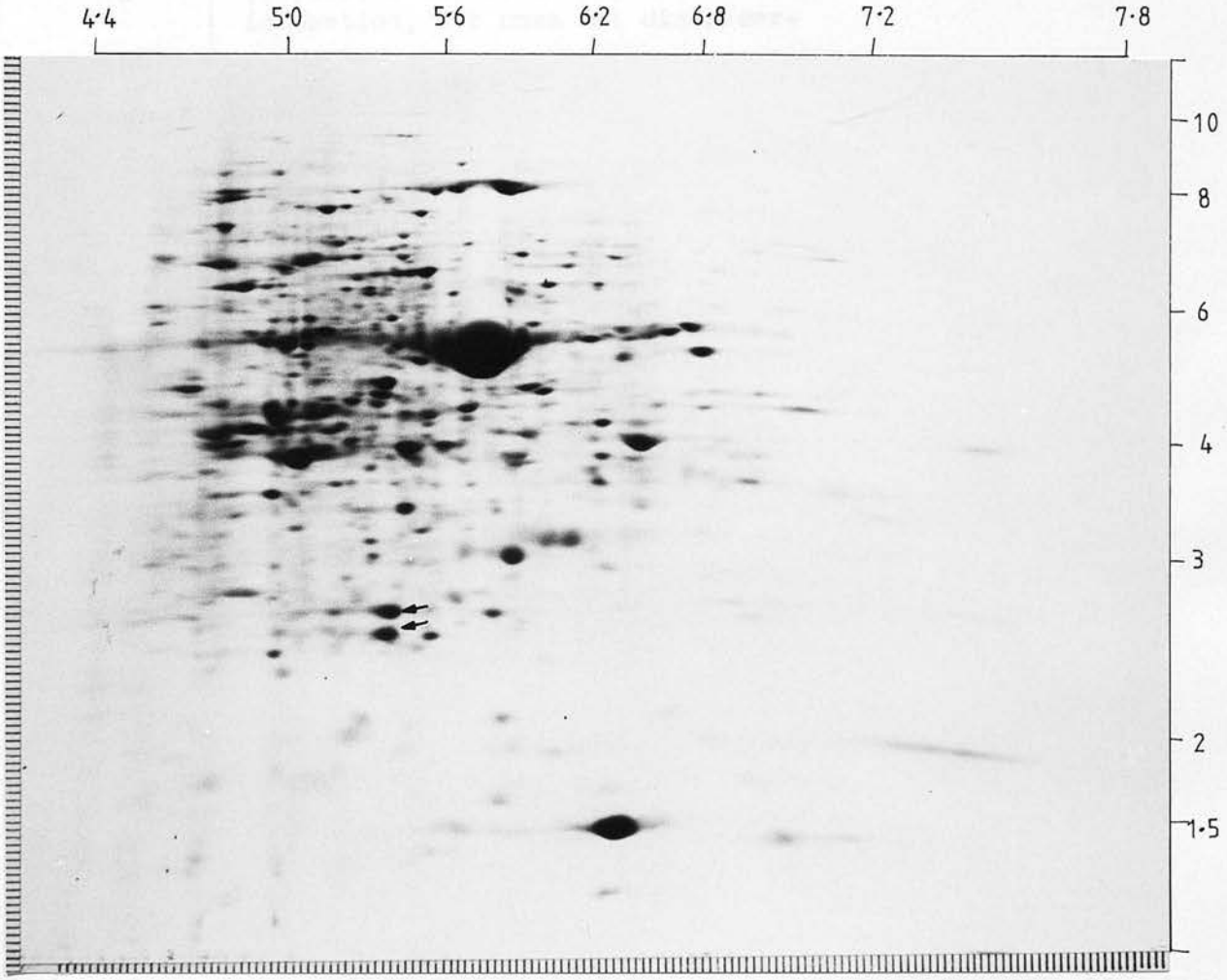

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

Table 9: Catalogue of in vivo labelled proteins in the mother fronds of S. polyrrhiza (N) incubated in 1 x 10^{-7} M ABA

Protein	Position & Occurrence
11	(14,500; 5·91) decreases after 1 day in ABA and remains very low thereafter.
12	(26,500; 5·83) decreases after 1 day in ABA and throughout incubation, but does not disappear.
13	(25,000; 5·53) increases after 3 days and thereafter.
23	(70,000; 5·55) appears day 1, increases day 3, and remains high thereafter.
35	(27,000; 5·98) increases throughout incubation.
47	(76,000; 5·56) increases at day 1 and remains constant thereafter.
54	(34,000; 5·06) increases throughout incubation.
74	(23,200; 5·53) increases throughout incubation.
124	(29,500; 6·18) disappears after 2 days in ABA.
125	(42,500; 6·22) decreases after 1 day and thereafter to a low level at 7 days.

Figures in brackets indicate the molecular weight in daltons and the isoelectric point of the protein respectively.

Figure 59

Pattern of *in vivo* labelled proteins during the development of the vegetative frond of *S. polyrrhiza* (N). Tissue was pulse labelled for 3 hours with ^{35}S methionine and soluble protein was extracted and loaded onto 2-dimensional poylacrylamide gels. Equal amounts of protein were applied to each gel (25 µg). Labelled proteins were visualised by fluorography.

a) Fronds $\leq$ 1 mm 18,000 cpm

b) fronds $\leq$ 2 mm 19,000 cpm

c) fronds $\leq$ 3 mm 17,000 cpm

d) fronds $\leq$ 4 mm 16,000 cpm

e) fronds $\leq$ 5 mm 16,000 cpm

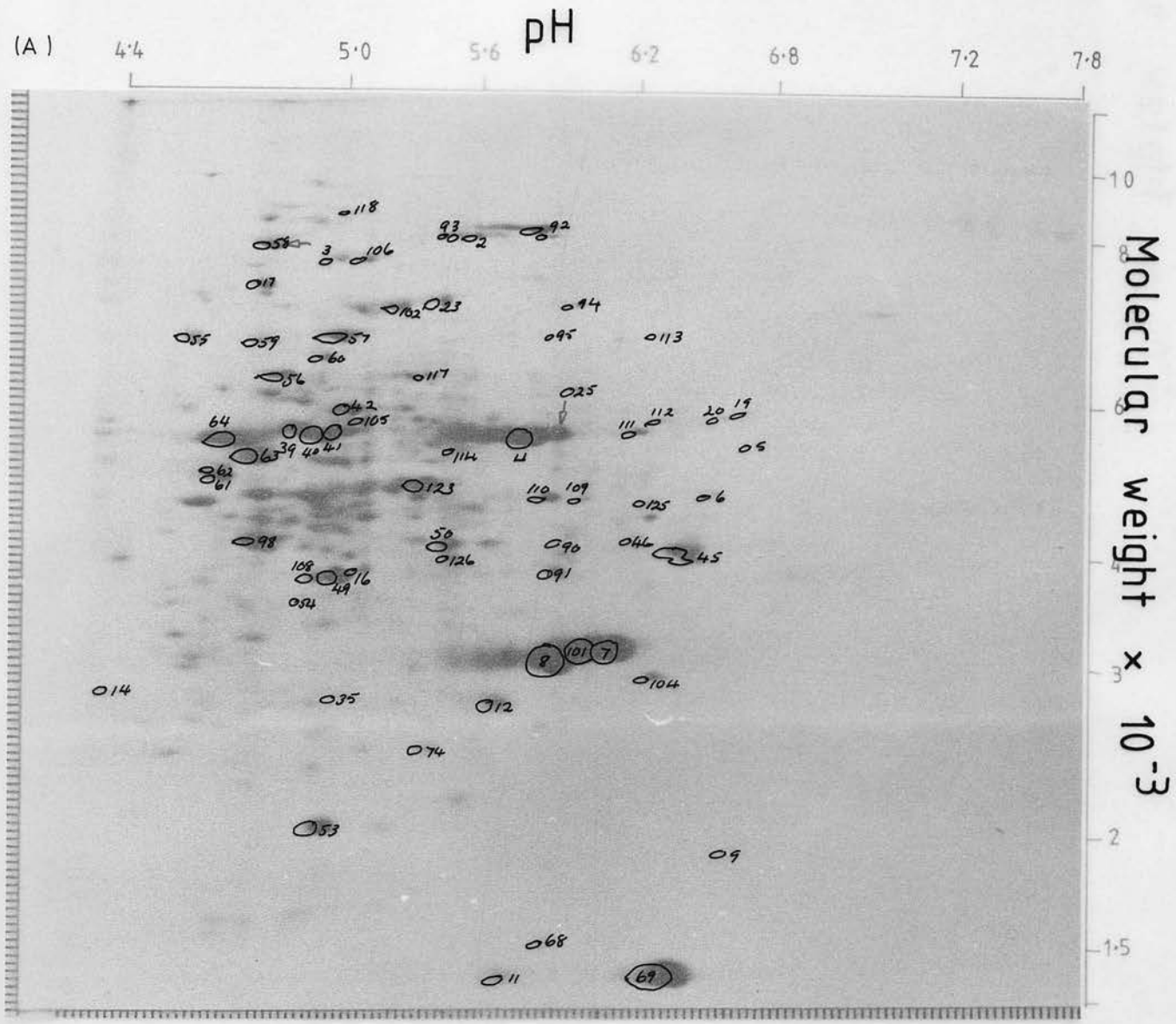
f) fronds $\leq$ 6 mm 14,000 cpm

g) fronds $\leq$ 7 mm 11,000 cpm

↙ indicates that protein has increased in amount from the day before

↗ indicates that protein has decreased in amount on the next day

FIGURE 59



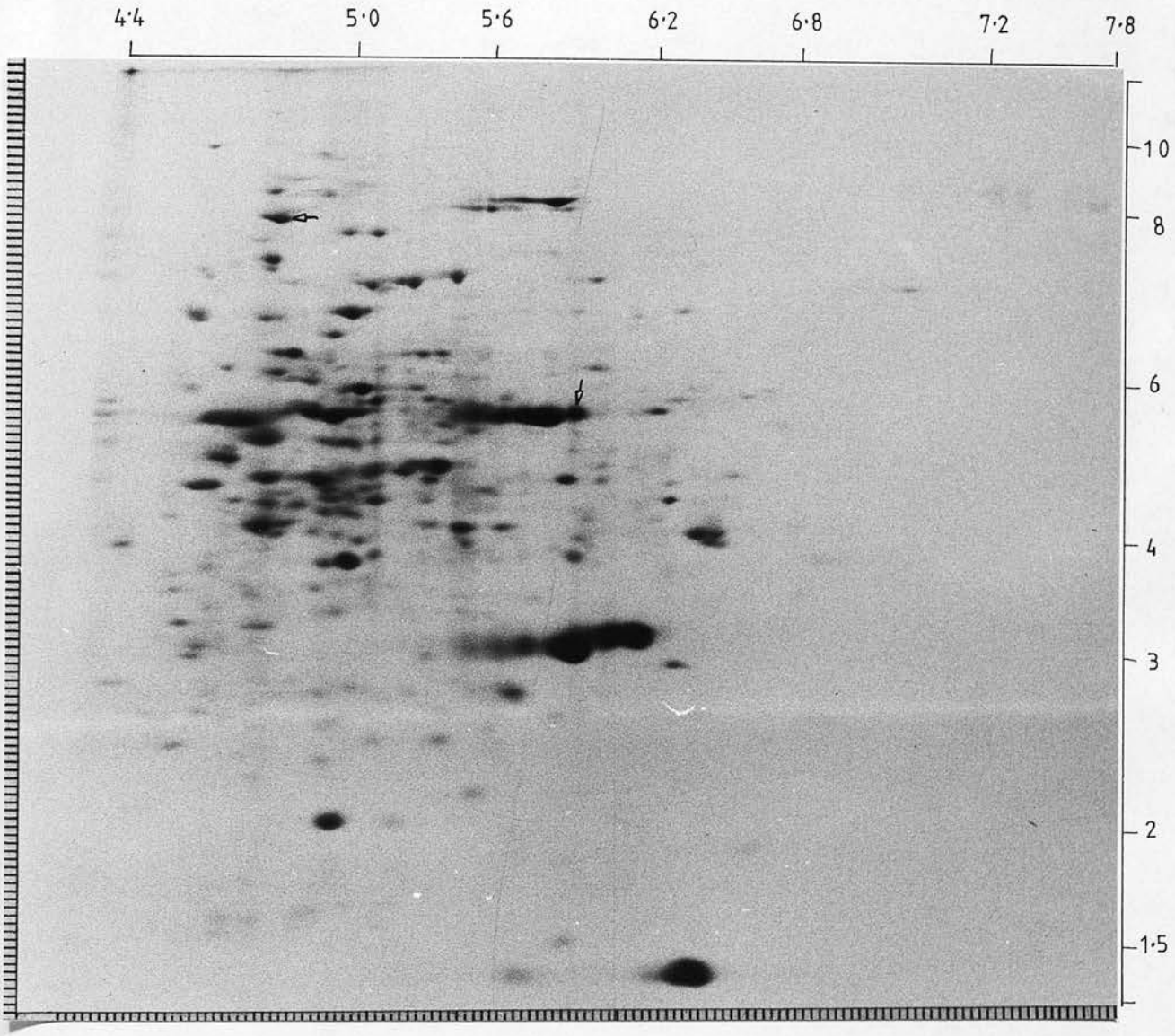
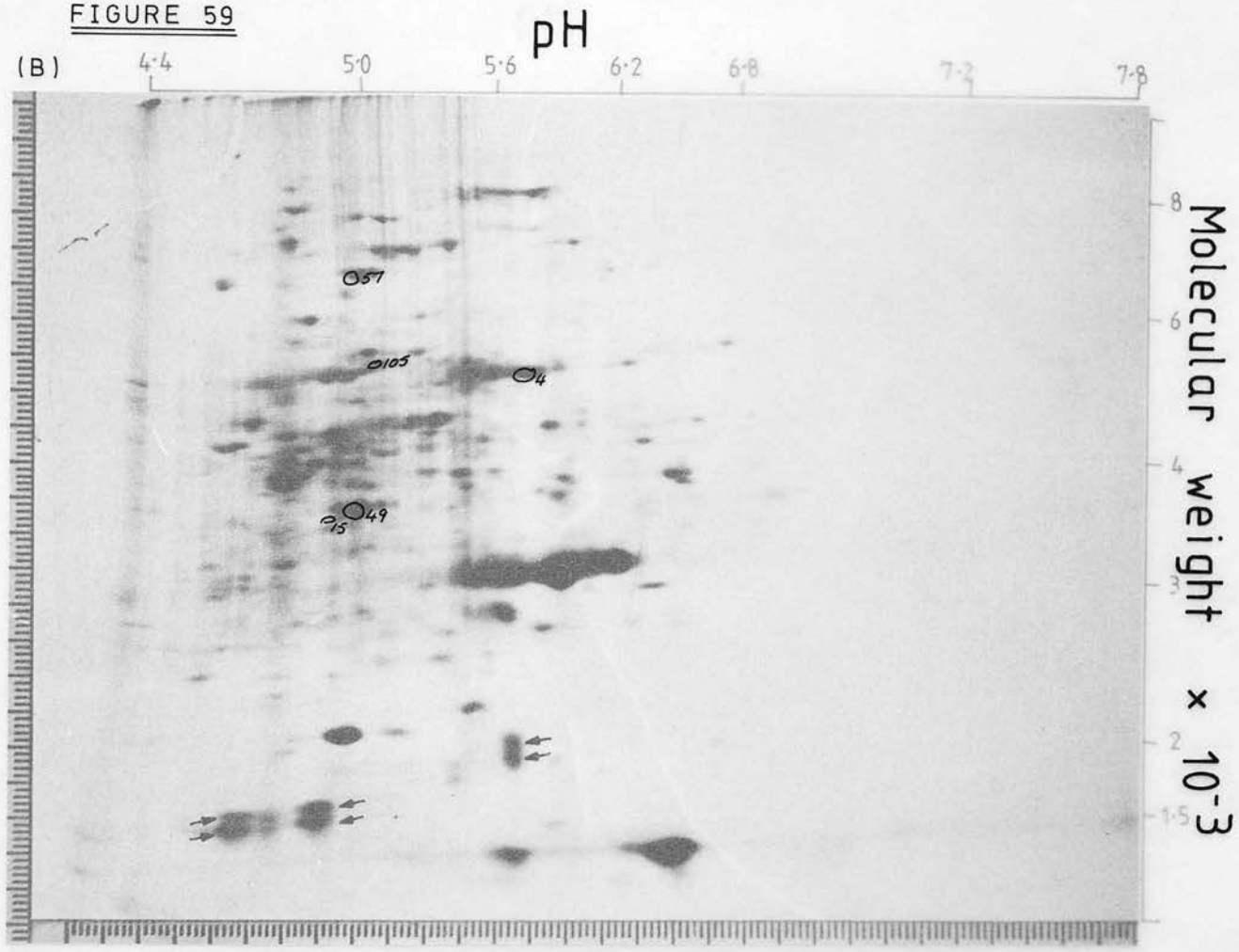
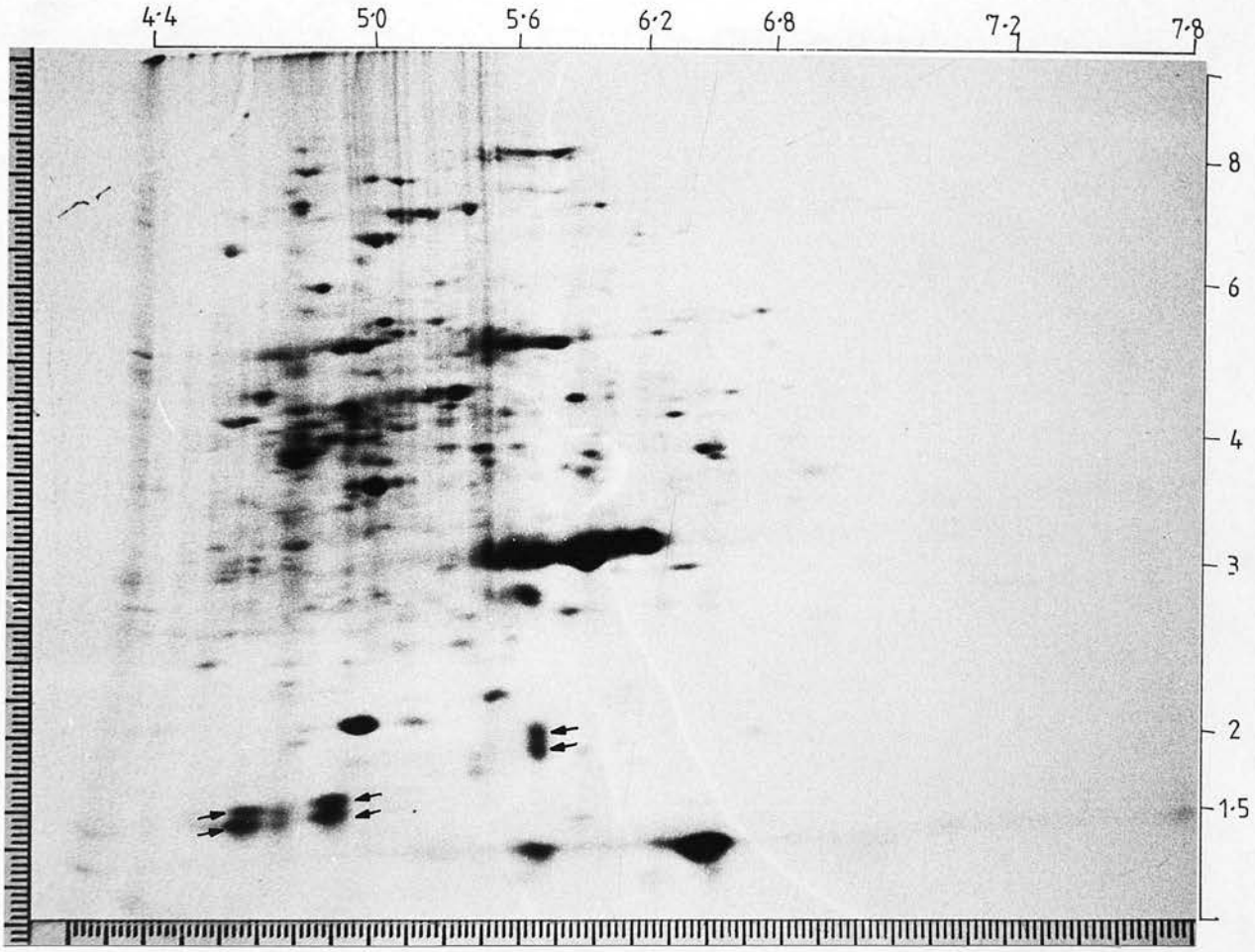

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 59


(B)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
8
6
4
3
2
1·5
O57
O105
O4
O15
O49


(C)
O57
O105
4
O15
O49


4·4
5·0
5·6
6·2
6·8
7·2
7·8
8
6
4
3
2
1·5

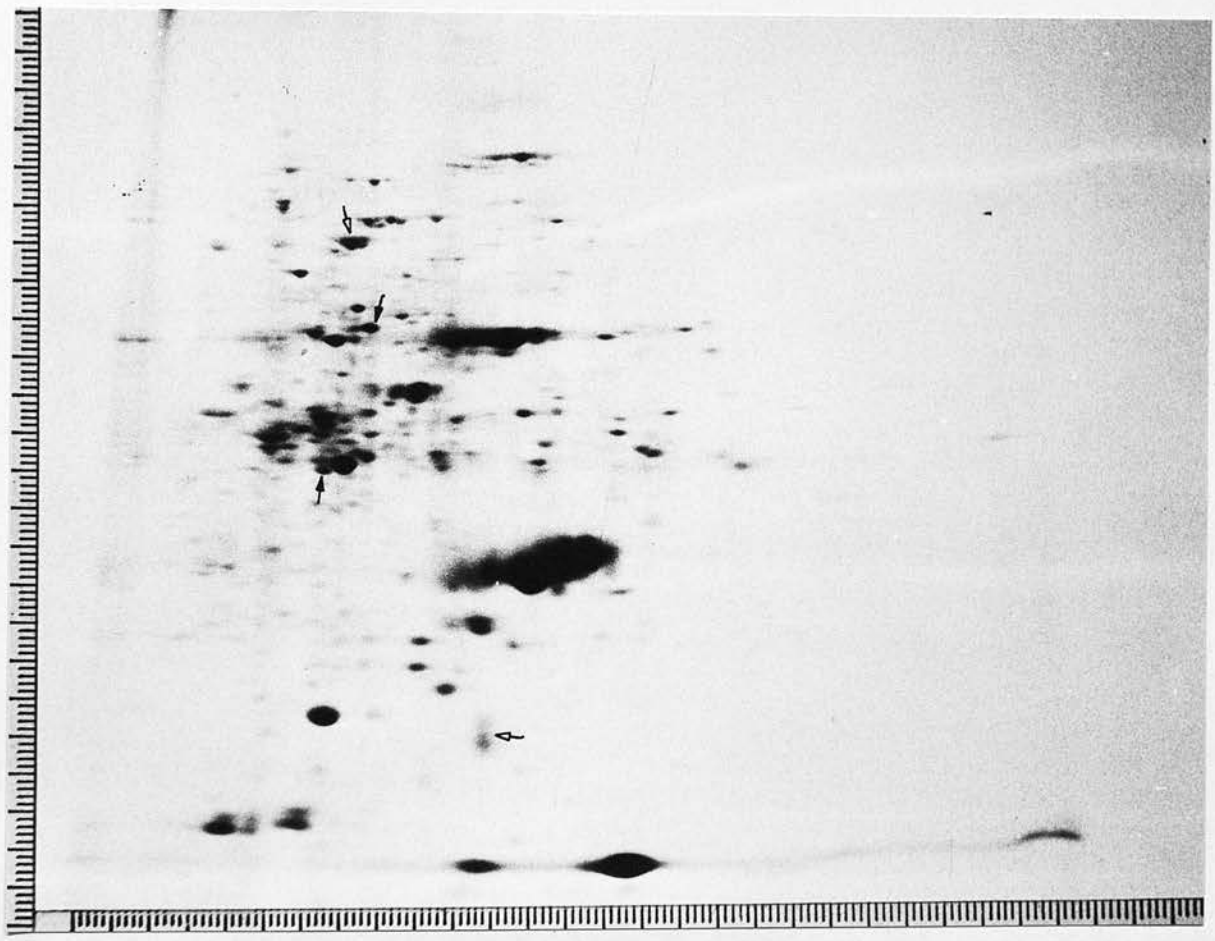
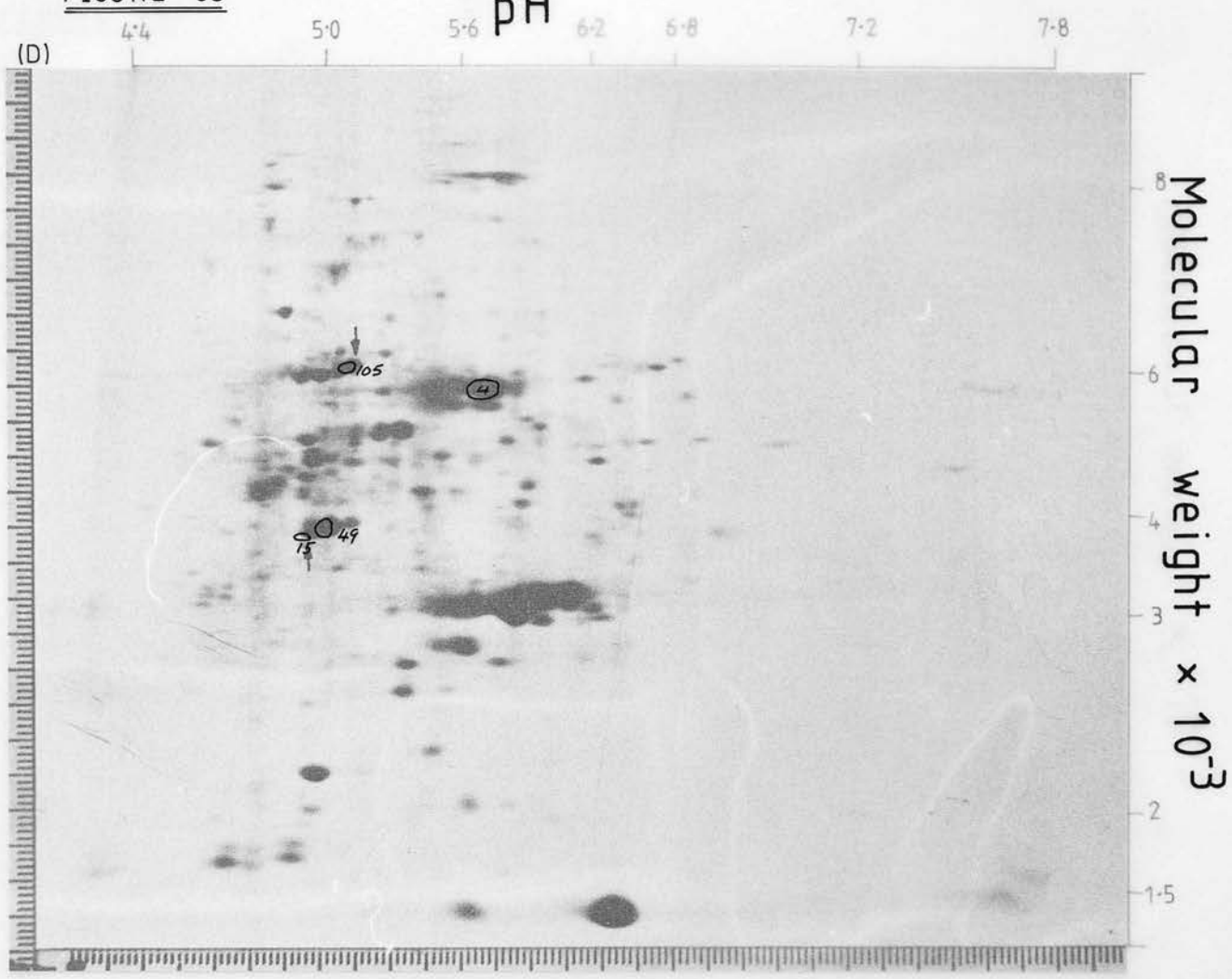
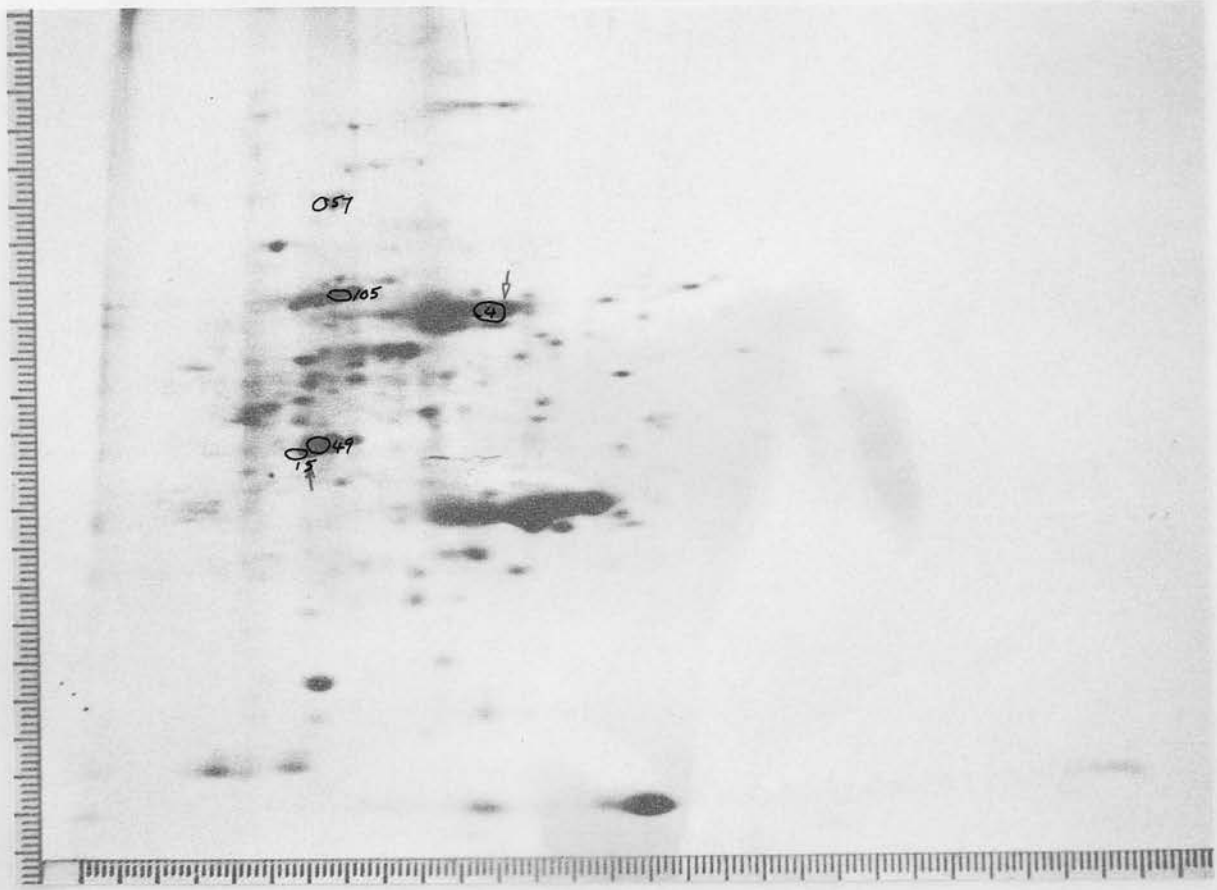
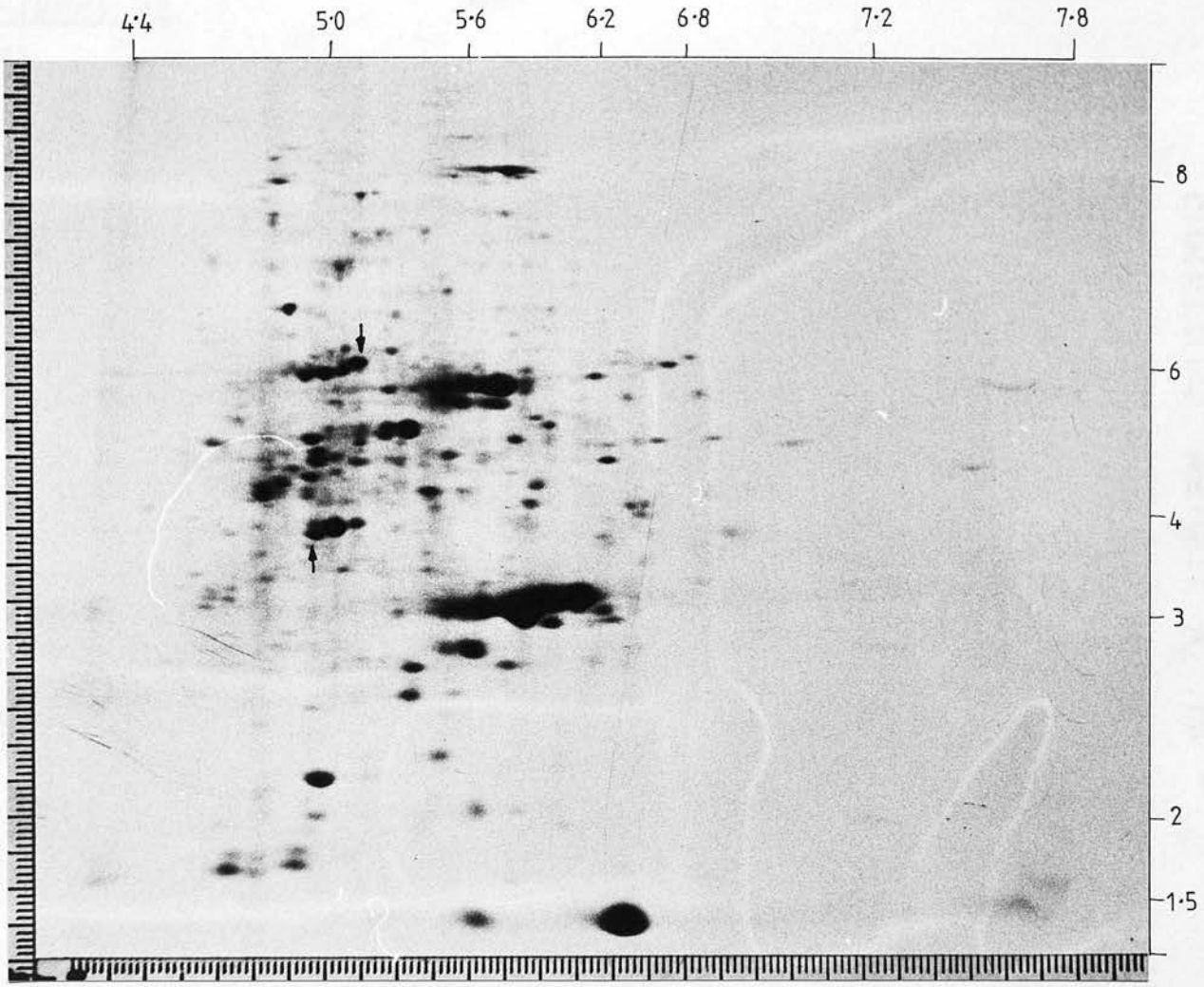


FIGURE 59


pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
(D)
Molecular weight × 10⁻³
8
6
4
3
2
1·5
105
4
049
15


(E)
057
105
4
049
15


4·4
5·0
5·6
6·2
6·8
7·2
7·8
8
6
4
3
2
1·5

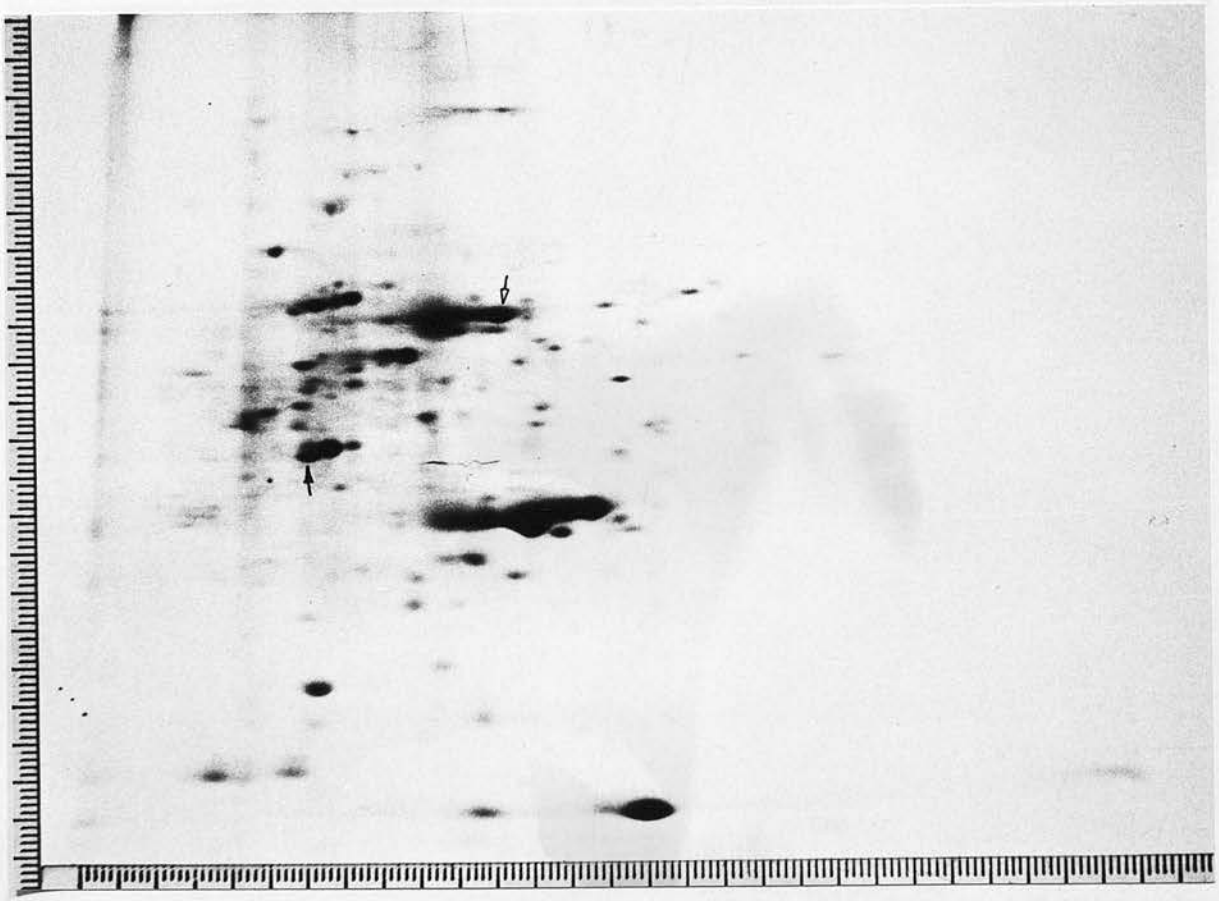
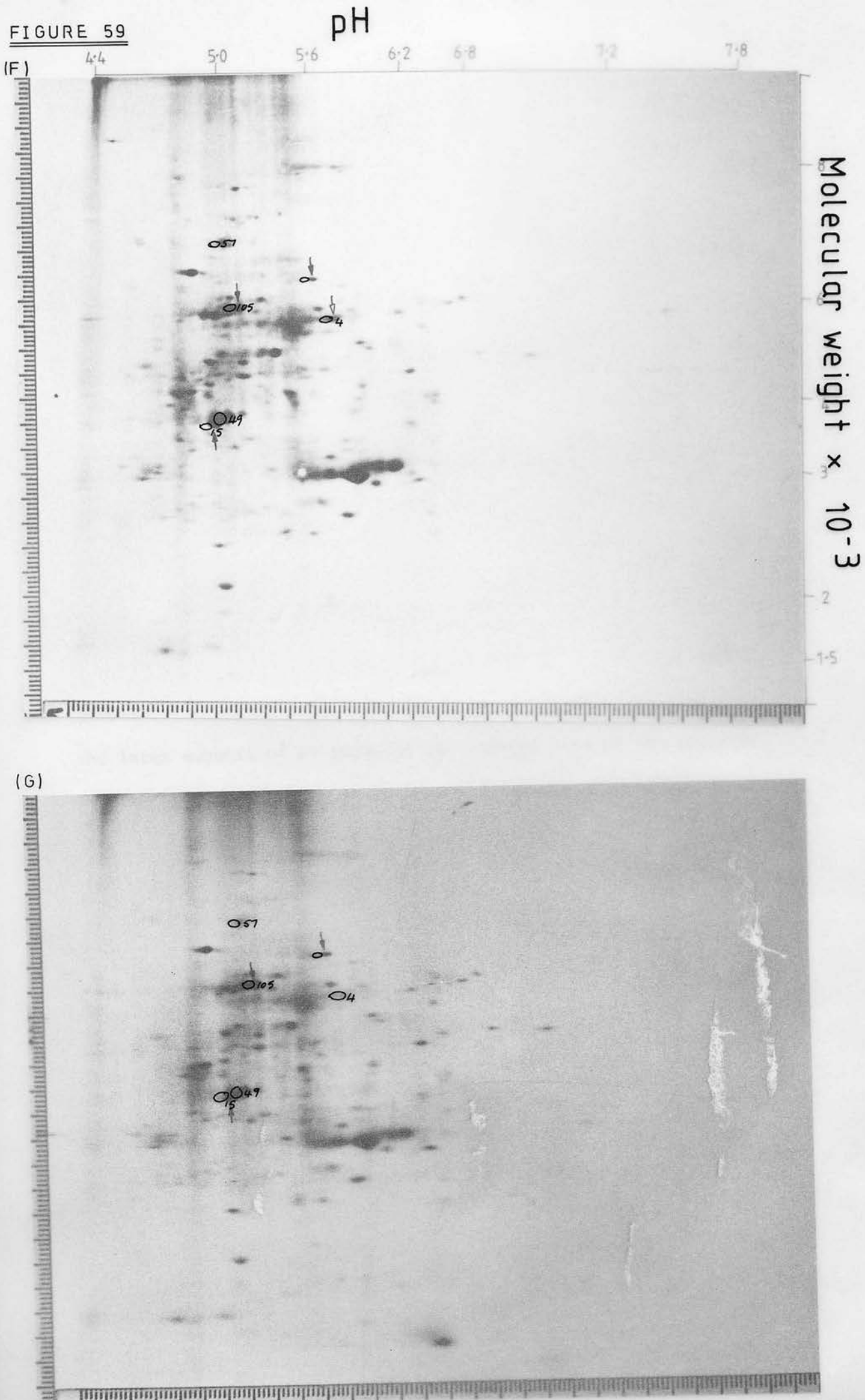
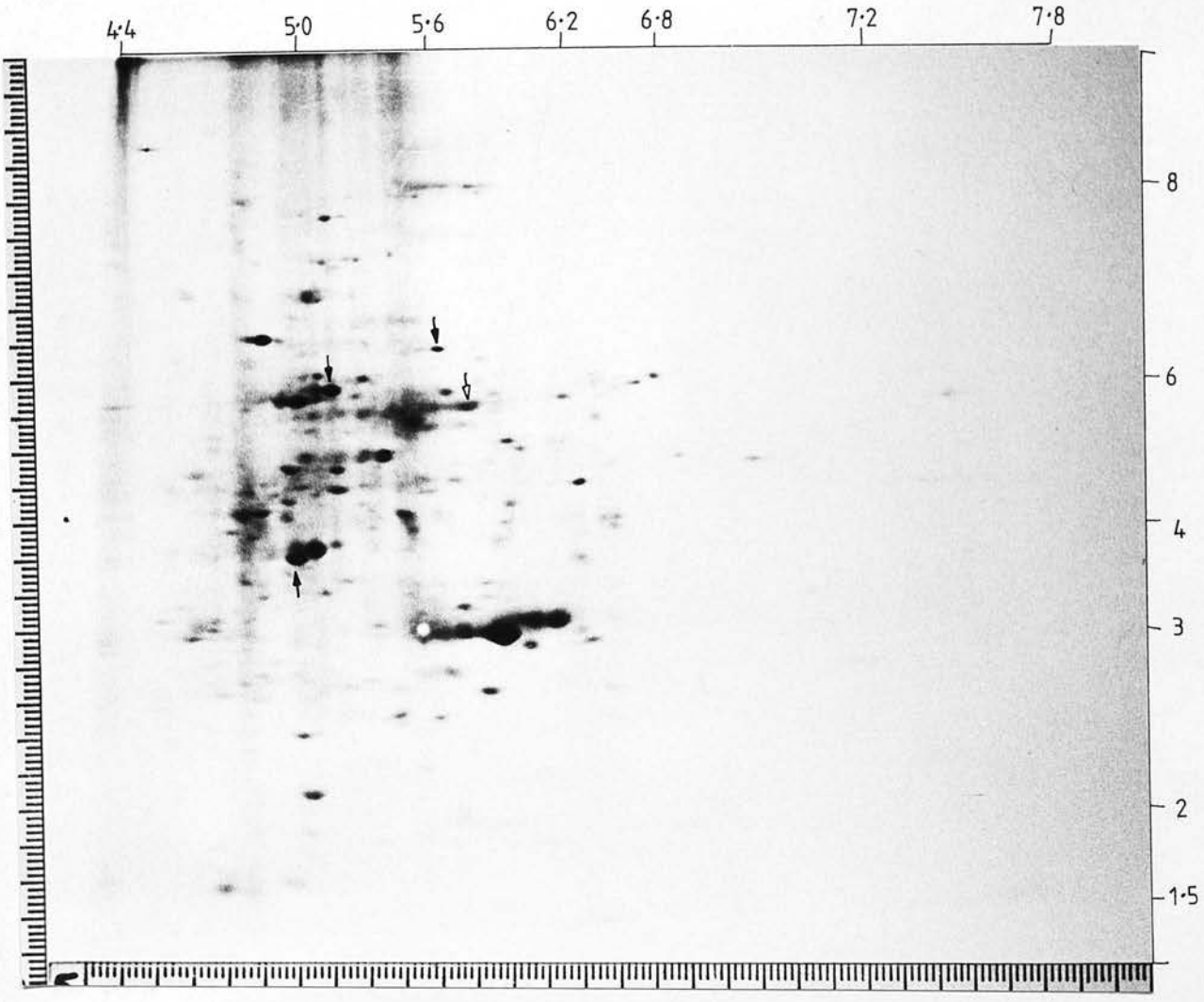
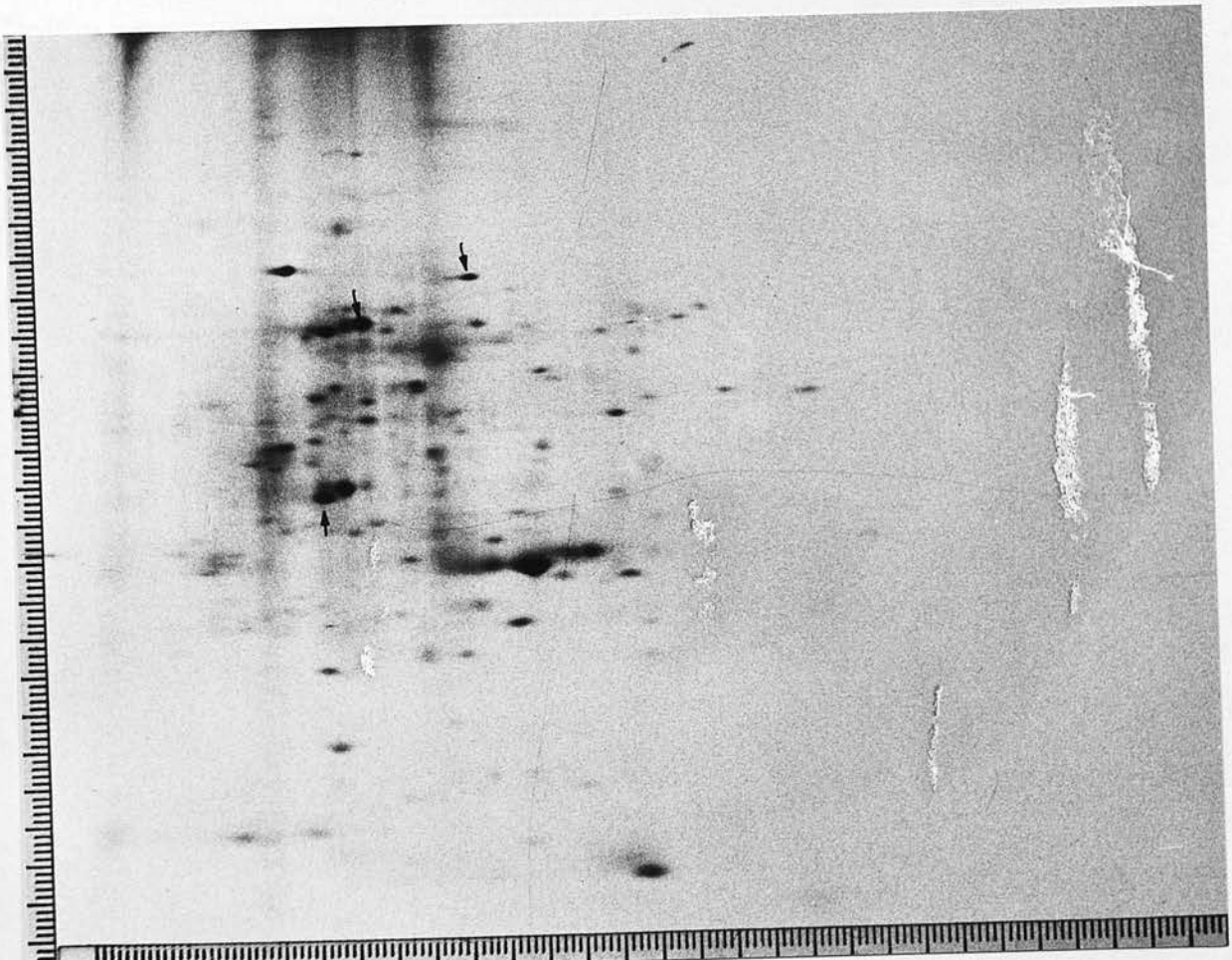


FIGURE 59


pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
(F)
Molecular weight × 10-3
8
6
4
3
2
1·5
O57
O
O105
O4
O49
O15
(G)
O57
O
O105
O4
O49
O15


4·4
5·0
5·6
6·2
6·8
7·2
7·8
8
6
4
3
2
1·5



the process of turion formation, since it is not shown during the development of the vegetative frond (Fig. 59) i.e. it is not a component of the normal maturity programme.

Protein 35

This protein can be seen to increase its label after only 1 day in ABA in the developing turion, whereafter it remains highly labelled even after 7 days (Fig. 57g). However its synthesis was also increased in the associated mother fronds (Fig. 58).

Protein 4

The synthesis of this protein decreases steadily during turion formation (Fig. 57). Its synthesis does not appear to be altered in the associated mother fronds (Fig. 58), but is decreased during the formation of the normal vegetative frond (Fig. 59). It is possible that the decline in the synthesis of this peptide (which is probably the large subunit of F1 protein) is a normal part of the maturity programme, not only of the vegetative frond but also of the turion. In this respect this change does not represent a unique effect characteristic of turion formation as does the decrease in protein 8. Another problem encountered with this protein was its unexplained disappearance from gels where it was expected to be seen e.g. day 4 ABA mother fronds (Fig. 58e). Normally this protein is readily soluble and is the most abundant protein on the gels. Occasionally however this protein appears only in very low amounts on gels loaded with soluble protein, and when this occurs it is also found in fairly large quantities in the pellet fraction. It is not understood why this happened so unpredictably. Not only does this

protein appear in the pellet fractions,but the protein present in the soluble fraction probably did not enter the gels since large amounts of protein sometimes remained at the top of the isoelectric focusing gel after focusing. This was always found to be the case when a mixture of pH 5 - 7 and pH 3·5 - 10 ampholines was used for isoelectric focusing; and this method was abandoned. The use of a pH 3·5 - 10 gradient only,improved the results remarkably, although obviously did not completely overcome the problem.

Protein 40

None of the above problems applied to any of the other proteins investigated. This protein increased dramatically during turion formation (Fig. 57). It does not increase its label in the associated mother fronds (Fig. 58), neither is this increase associated with the normal development of the vegetative frond (Fig. 59). The increase in the synthesis of this protein therefore seems to be restricted to the action of ABA on young tissue capable of forming turions only.

Protein 54

This protein's label increases from being just noticeable at day 0 to becoming one of the most prominent proteins of the 7 day old turion (Fig. 57). It also increases in the associated mother fronds (Fig. 58). Unlike protein 8, this protein appears to be induced by ABA in both tissues and is not peculiar to the developing turion. Additional evidence for the induction of the increase in synthesis of protein 54 by ABA comes from the fact that this protein is barely visible throughout the development of the vegetative frond

i.e. no ABA, no protein 54.

Protein 45

Protein 45 increases throughout turion formation (Fig. 57). It shows no change in the associated mother fronds (Fig. 58). Protein 45 decreases slightly during the development of the vegetative frond (Fig. 59).

Protein 49

This protein remains high during turion formation (Fig. 57) and is highly labelled in the 7 day old turion (Fig. 57g). It remains unchanged in the mother fronds associated with the developing turions (Fig. 58) and during the development of the vegetative frond (Fig. 59).

Protein 53

This protein disappears 7 days after ABA addition in the developing turion (Fig. 57g). Protein 53 does not change in the associated mother fronds but declines slightly during the development of the vegetative frond (Fig. 59)

Proteins 42 and 105

These proteins increase throughout turion formation (Fig.57), 105 more than 42. They show no change in the mother fronds (Fig. 58) but 105 increased during the development of the vegetative frond (Fig. 59).

Proteins 13 and 74

Protein 13 is lost during turion formation and 74 decreased (Fig. 57). Both these proteins increase in the associated mother fronds (Fig. 59)

Protein 124

This protein increases during vegetative frond development (Fig. 59). It is present in the mother fronds but disappears soon after ABA treatment. Protein 124 does not appear in the developing turions.

Protein 125

This polypeptide decreases after 1 day in ABA in the mother fronds and is present at a very low level after 7 days incubation in ABA (Fig. 58). It does not decrease significantly during turion formation or during vegetative frond development.

Protein 11

This protein also decreases after only 1 day in the mother fronds and remains low thereafter (Fig. 58), although it also decreased during turion formation and vegetative frond development (Figs. 57 and 59)

Protein 12

Protein 12 decreased throughout turion formation (Fig. 57) and in the associated mother fronds (Fig. 58), but did not change during vegetative frond development (Fig. 59).

Protein 15

This protein decreased during turion formation (Fig. 57) and disappeared after 7 days, while increasing during vegetative frond development (Fig. 59).

It is therefore apparent that at least 4 different classes of protein changes exist during the development of fronds of S. polyrrhiza:

1) Proteins that only alter during turion formation in the developing turion e.g. 7, 8, 15, 40, 42, 45, 49, 53, and those which change in the opposite direction to the changes found in the mother fronds e.g. 13, 74.
2) Proteins which change in all ABA treated tissues e.g. 11, 12, 15, 35, 54.
3) Proteins where the change is associated with the maturity of both developmental programmes e.g. 4, 11, 105, and those which change in the opposite direction in turion development to vegetative frond development e.g. 15, 45.
4) Proteins which only change in the mother fronds exposed to ABA e.g. 125, and maturity proteins which change only in the mother fronds e.g. 124.

These experiments have shown that the inhibition of protein synthesis which occurs during the formation of turions is by no means a non-specific one. Although the synthesis of a few major proteins and many minor ones is inhibited, the synthesis of other proteins has been enhanced relative to the total pattern. The change in the pattern of the proteins synthesised during turion formation in no way resembles the less striking change in the peptide pattern during the development of the vegetative frond.

The membrane proteins of the developing turion were also investigated and these are shown in Fig. 60 and are catalogued in table 10. The pattern of membrane proteins does not appear to change as obviously as the soluble proteins, and is characterised by a decrease in the synthesis of the majority of the polypeptides.

Figure 60

The pattern of *in vivo* labelled insoluble proteins during the development of the turion of *S. polyrrhiza* (N) in 1 x 10^{-7}M ABA. Tissue was pulse labelled for 3 hours in ^{35}S methionine and membrane protein extracted and loaded onto 2-dimensional polyacrylamide gels. Equal amounts of insoluble protein were loaded onto the gels (50 µg).

a) day 0 (untreated) 189,000 cpm

b) day 1 116,000 cpm

c) day 2 138,000 cpm

d) day 3 114,000 cpm

e) day 4 129,000 cpm

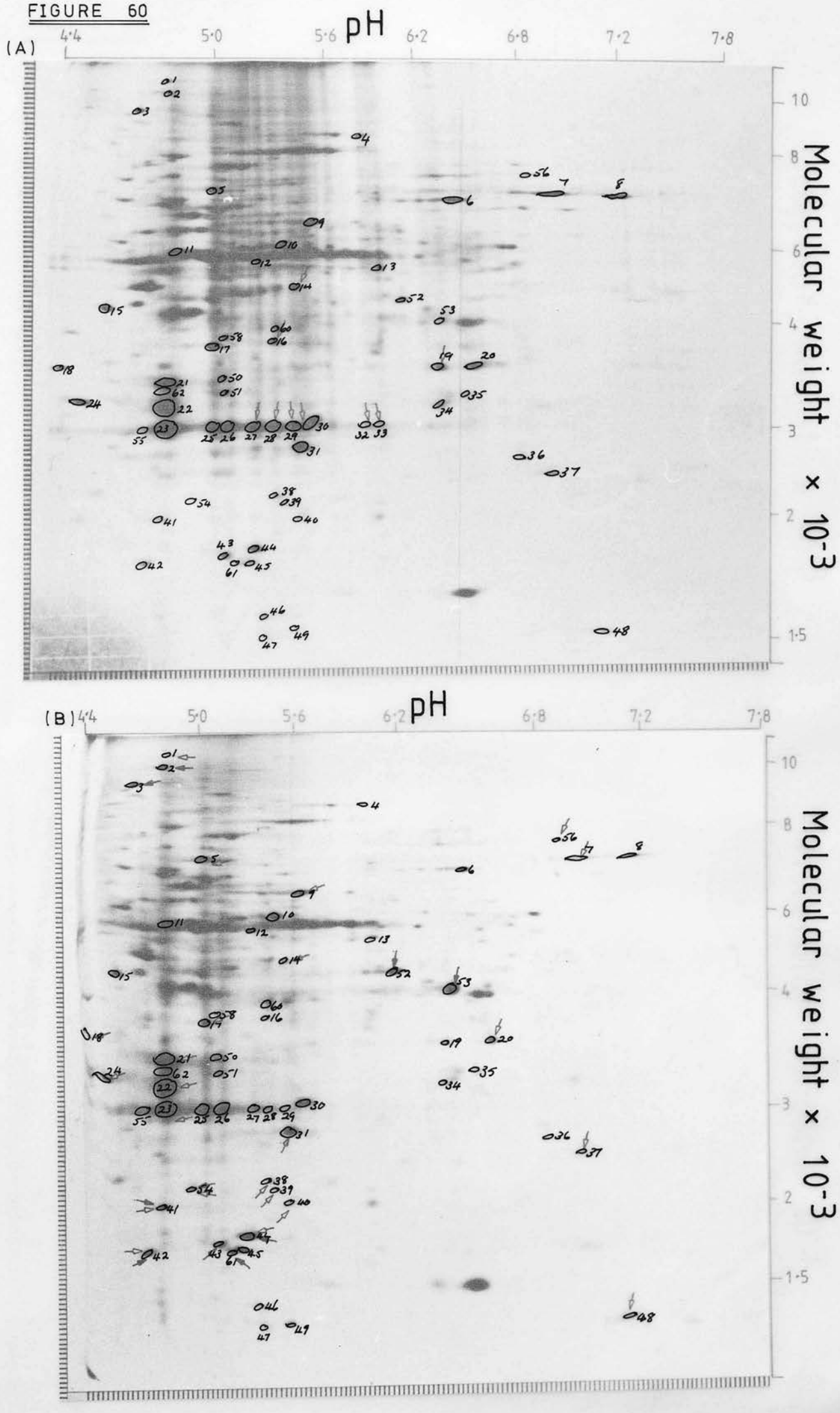
f) day 5 76,000 cpm

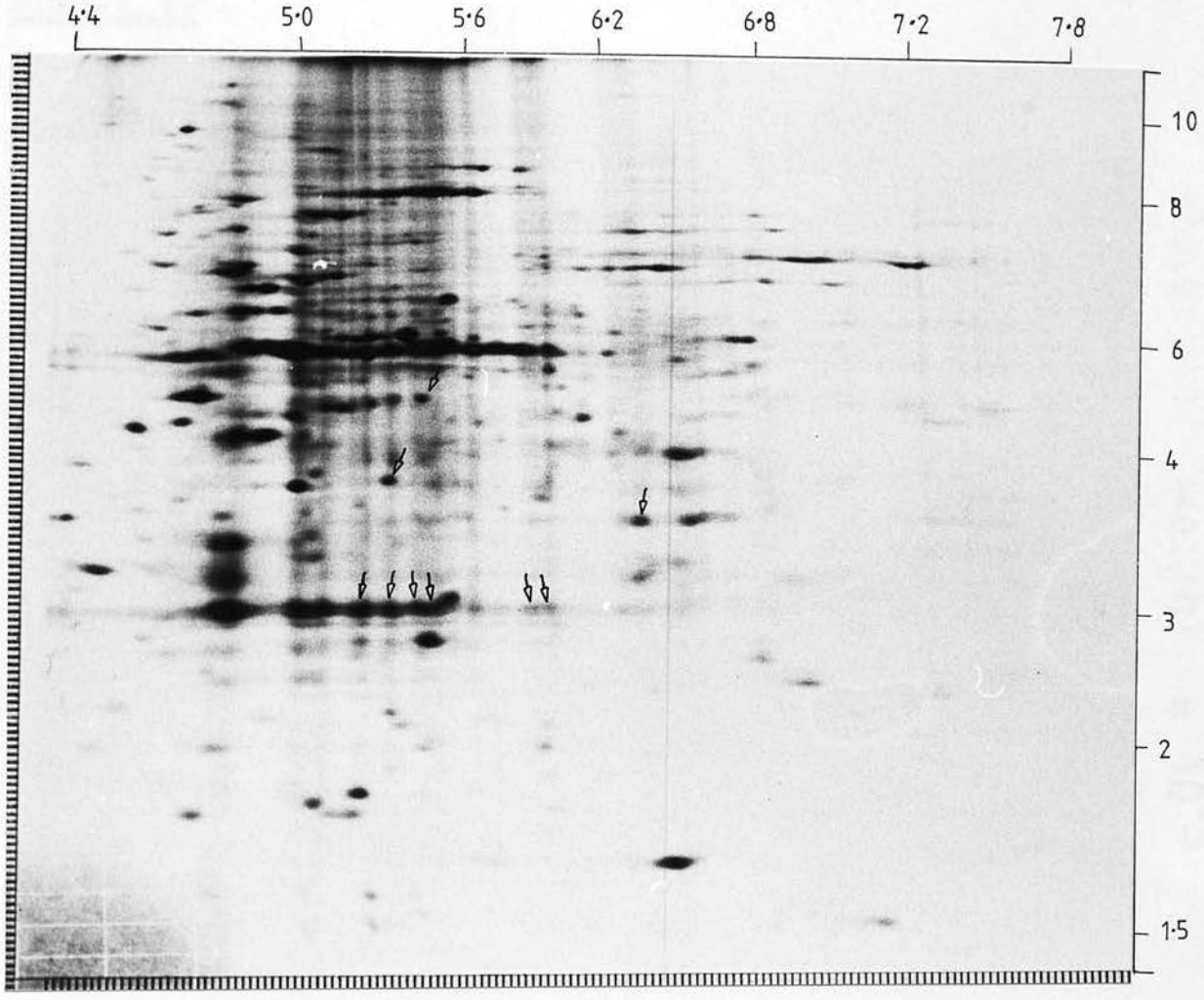
g) day 7 114,000 cpm

↙ indicates that the protein increases from the day before

↙ indicates that the protein decreases on the next day

FIGURE 60


(A)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5
(B)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight × 10⁻³
10
8
6
4
3
2
1·5


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

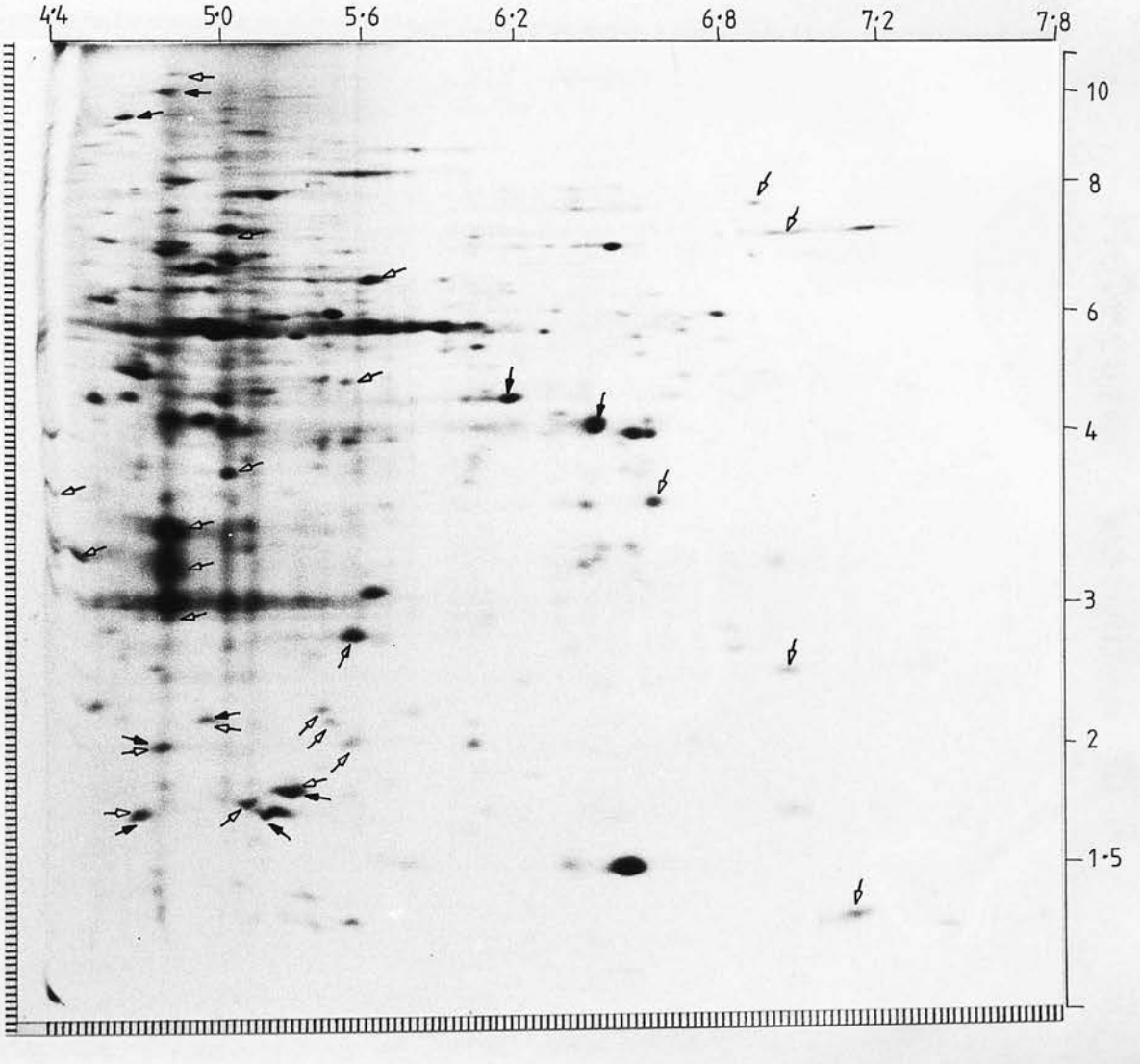
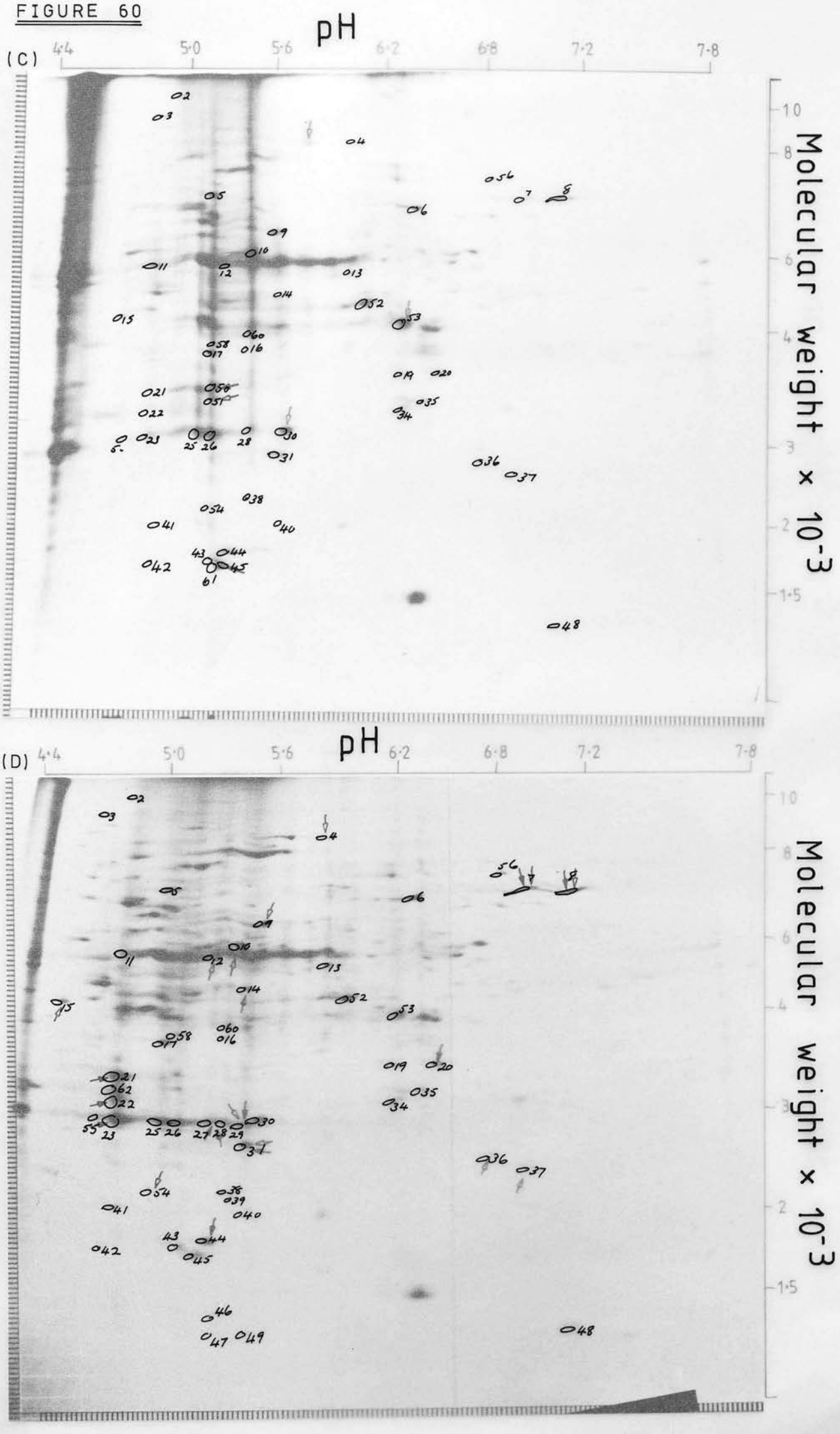
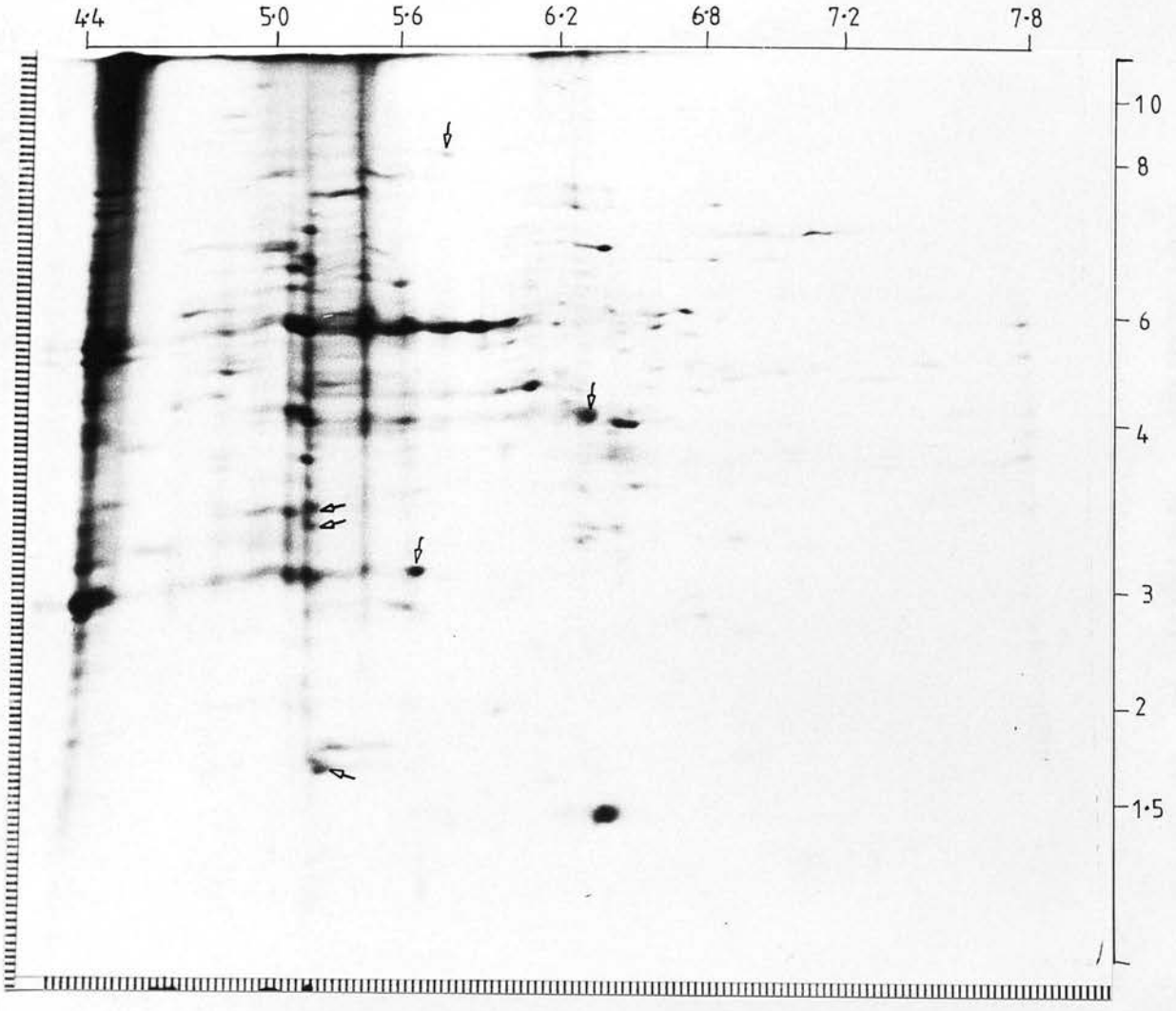

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 60


(C)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10⁻³
10
8
6
4
3
2
1·5
(D)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10⁻³
10
8
6
4
3
2
1·5


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

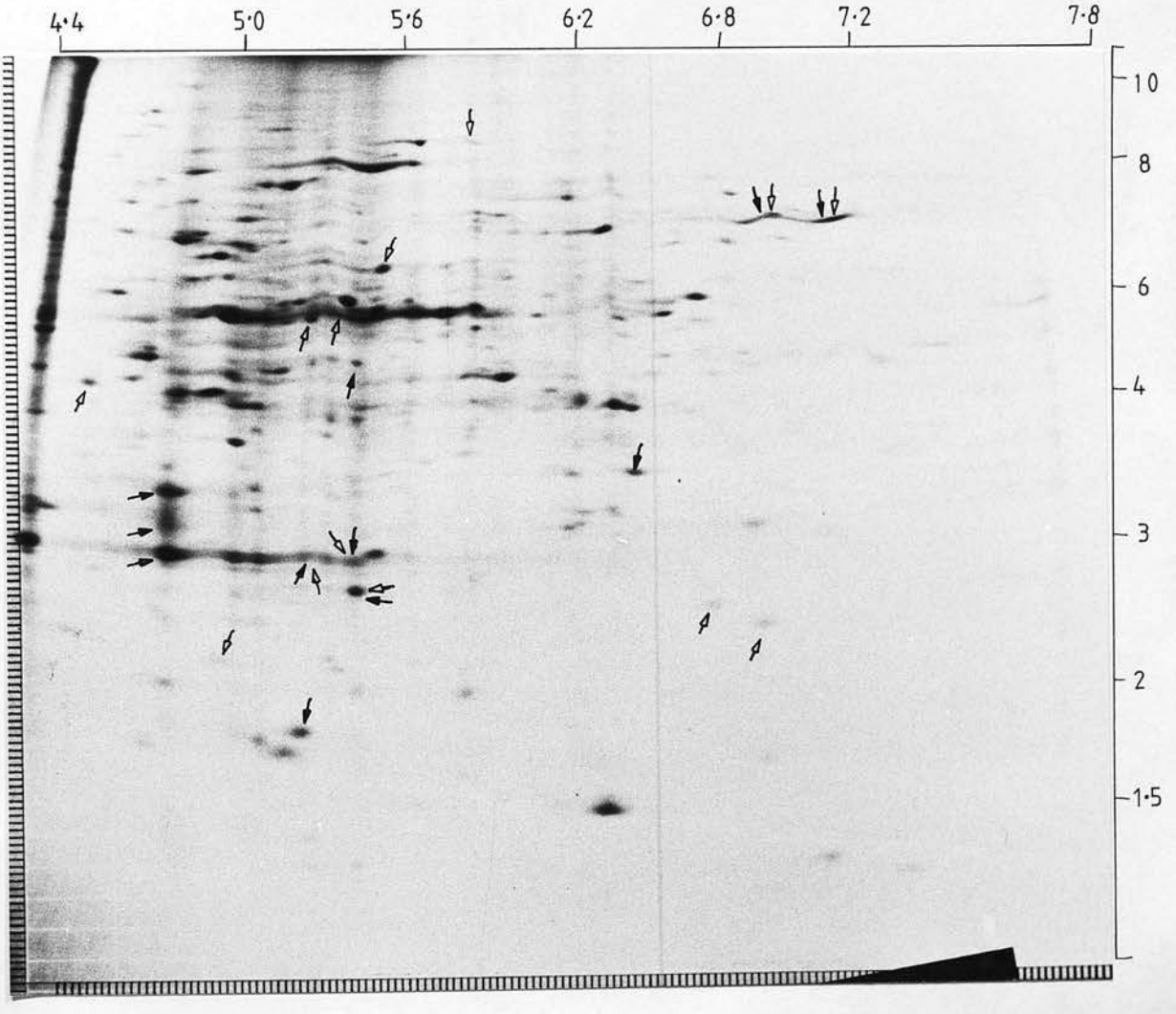
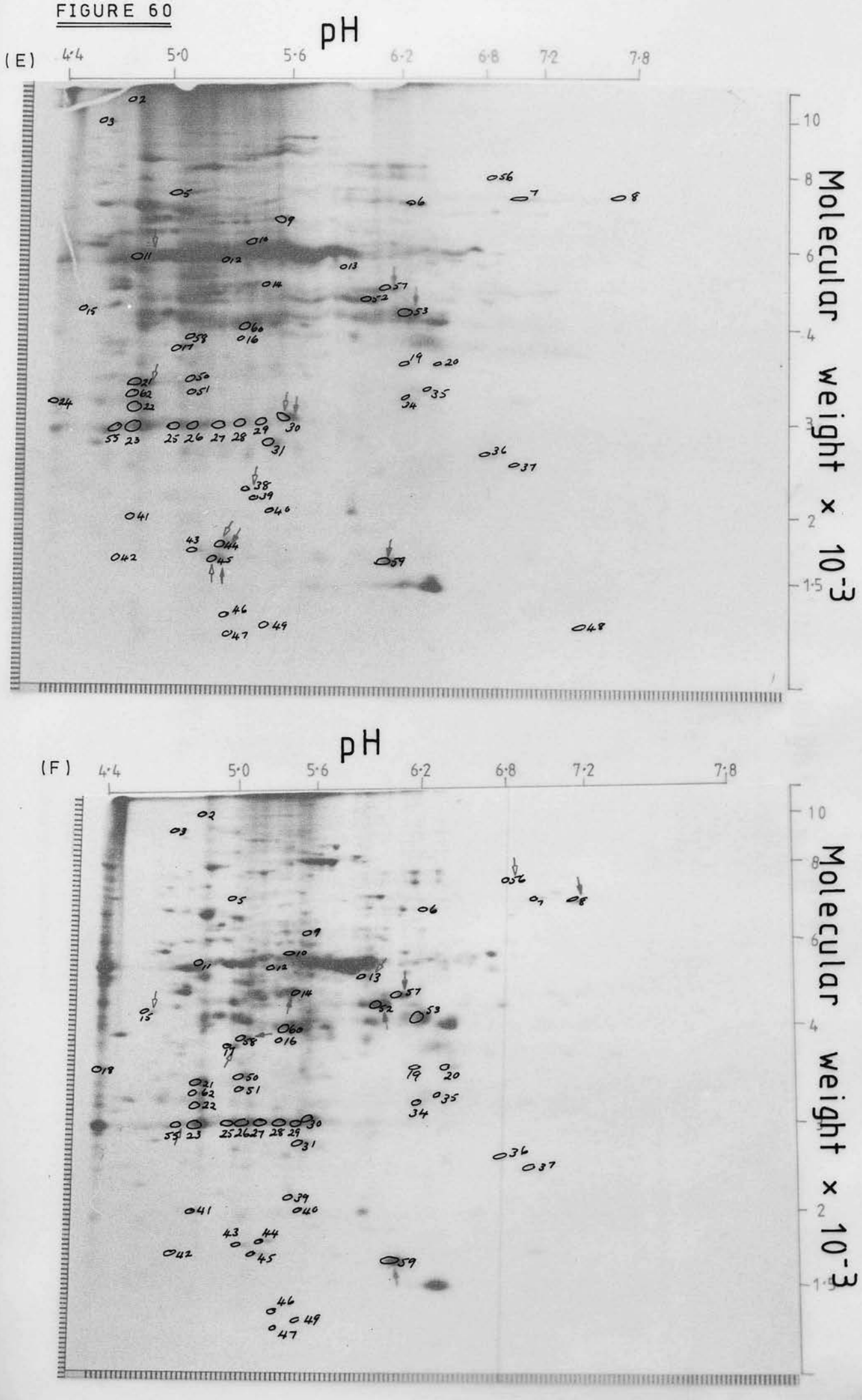
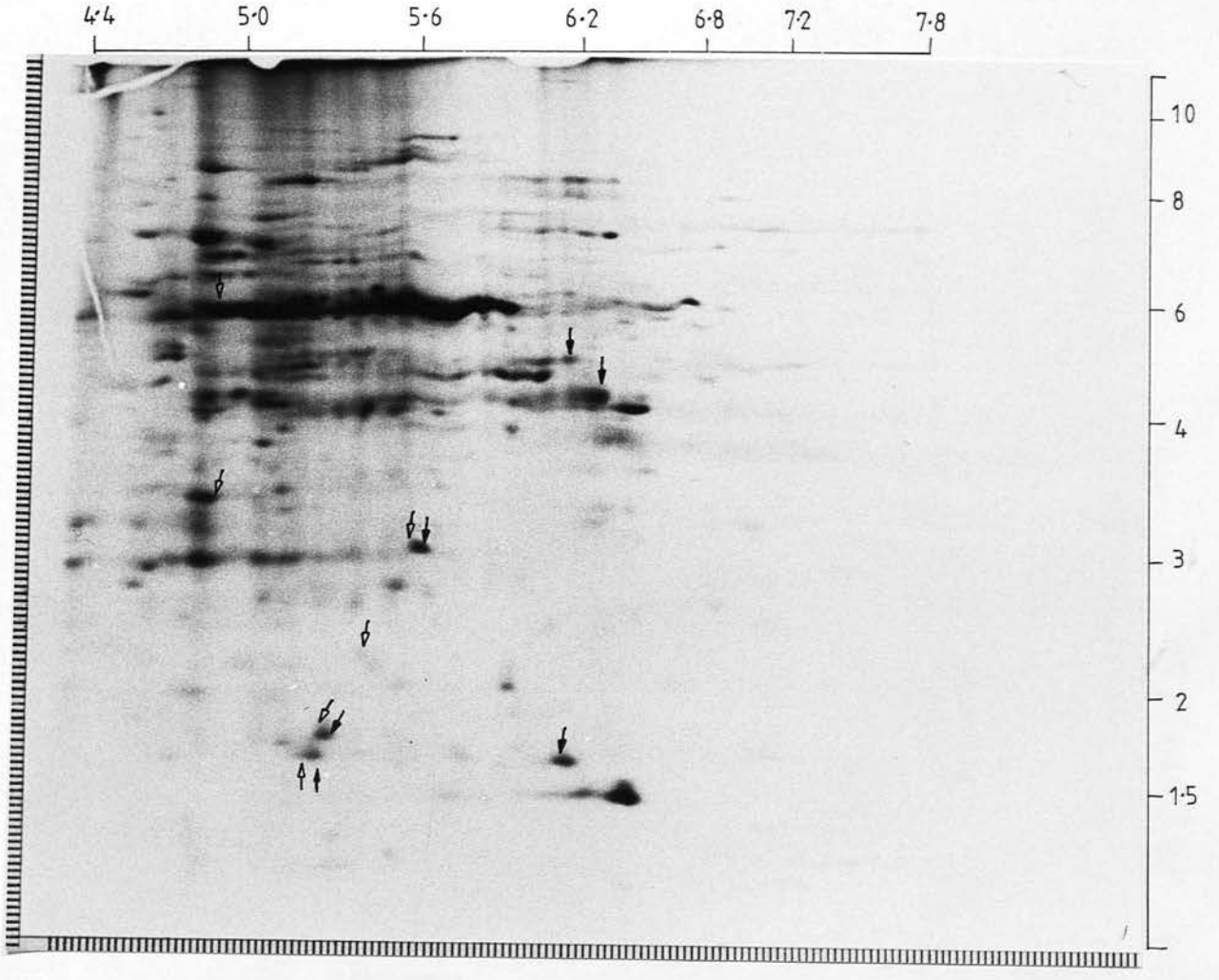

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 60


(E)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10-3
10
8
6
4
3
2
1·5
(F)
pH
4·4
5·0
5·6
6·2
6·8
7·2
7·8
Molecular weight x 10-3
10
8
6
4
2
1·5


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

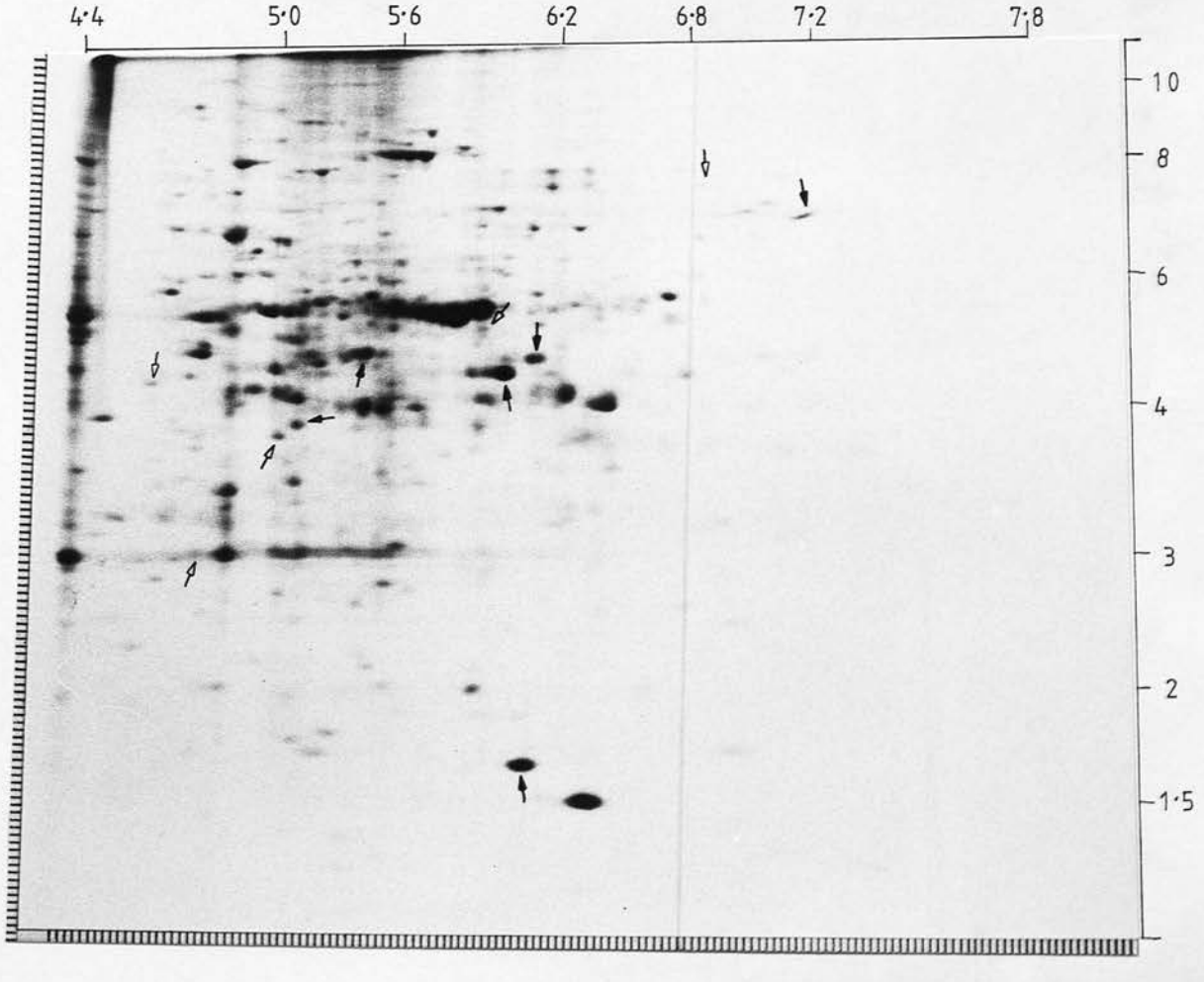
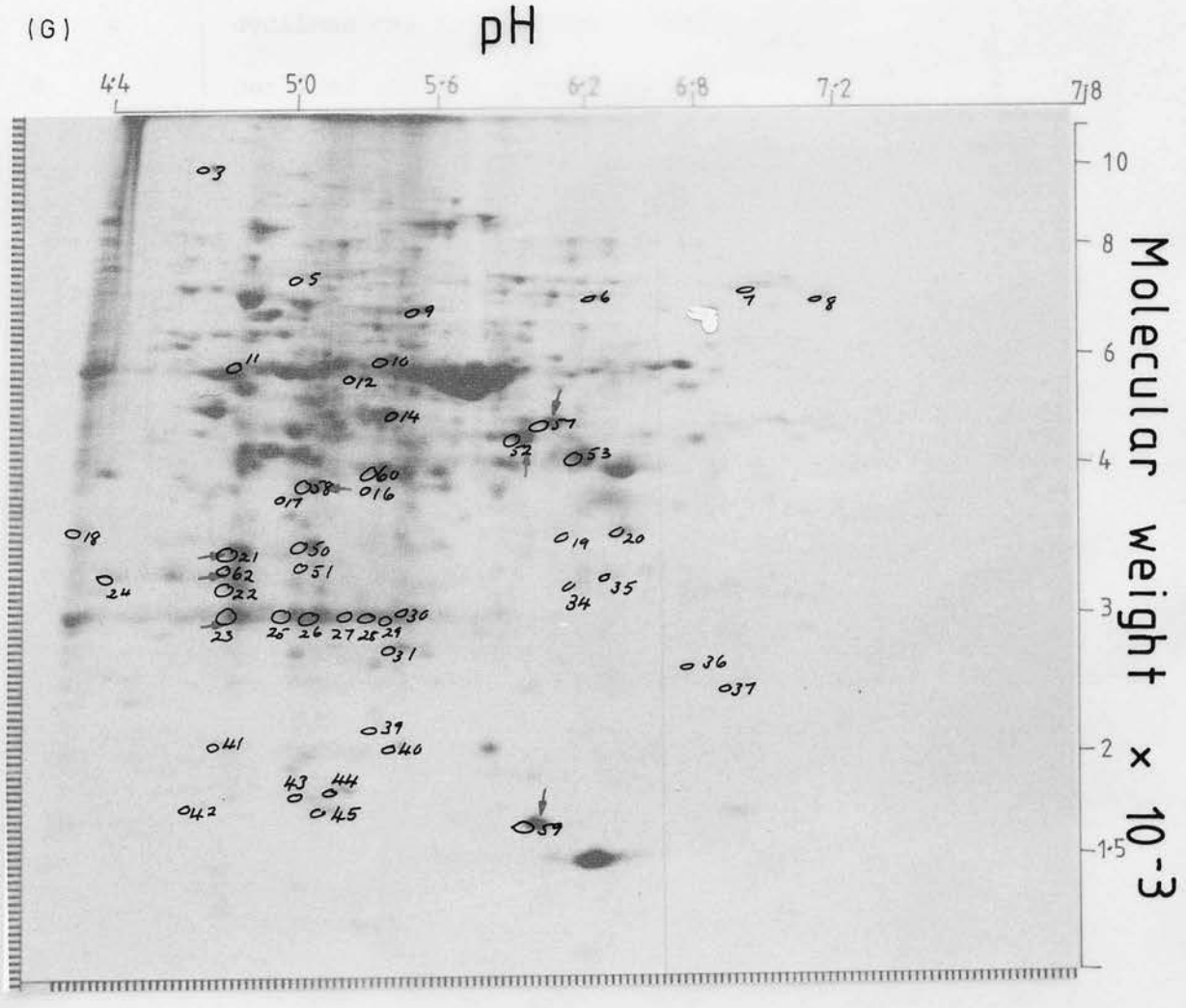

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

FIGURE 60



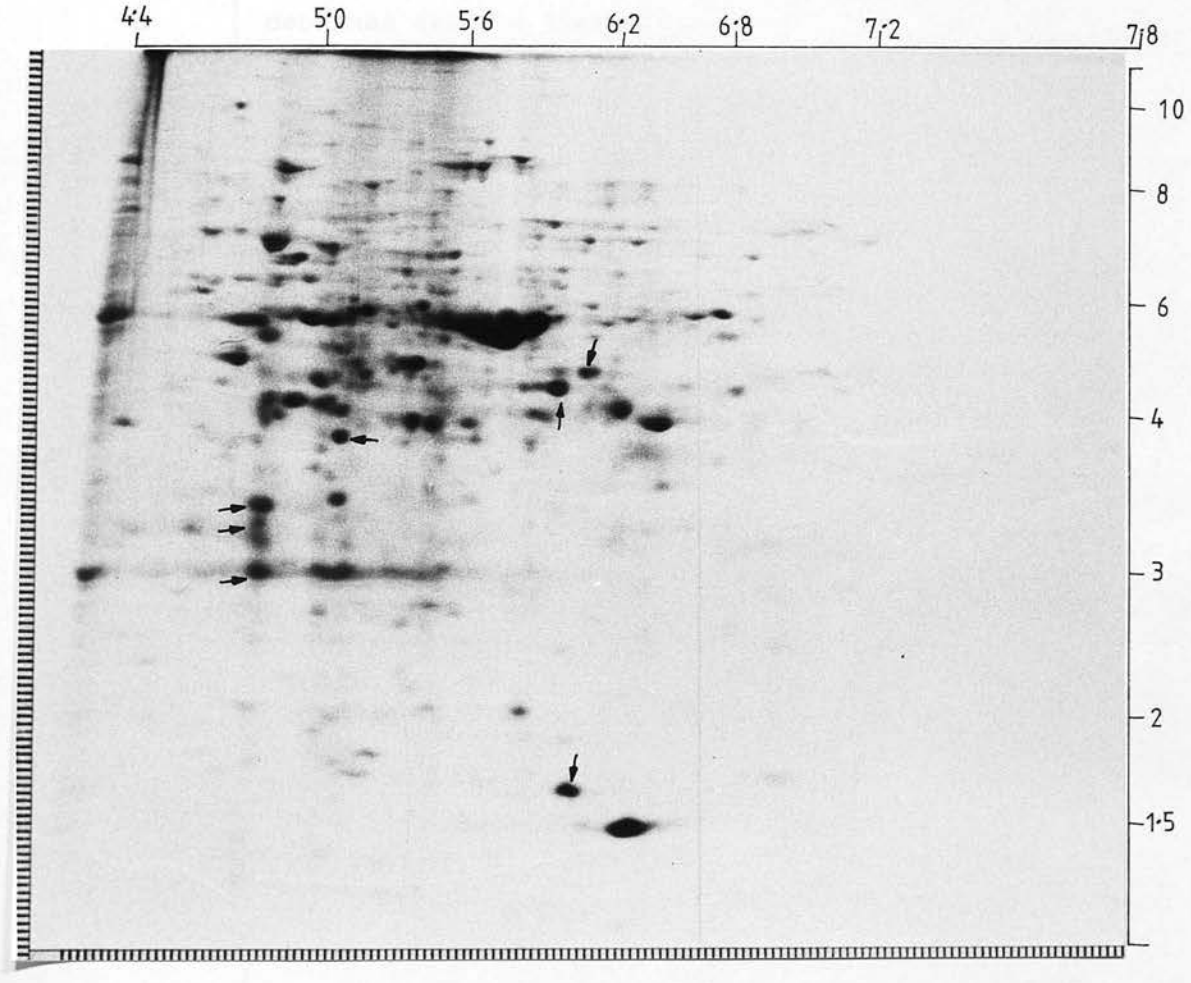

4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

Table 10: Catalogue of insoluble protein during the development of the turion of S. polyrrhiza (N) in 1×10^{-7} M ABA

	Protein	Occurrence
●	1	disappears day 2
●	2	declines day 2, disappears day 7
o	3	declines day 2
●	4	declines day 1, disappears day 4
o	5	declines day 3 & thereafter
*	6	remains constant
o	7	declines day 1 & thereafter
o	8	declines day 1 & thereafter
o	9	declines day 2 & thereafter
o	10	declines day 5
o	11	declines day 2 & thereafter
o	12	declines day 5 & thereafter
●	13	declines day 4 & thereafter, disappears day 7
o	14	declines day 1 & thereafter
●	15	declines day 2 & thereafter, disappears day 7
o	16	declines day 1 & thereafter
o	17	declines day 4 & thereafter
o	18	declines day 7
o	19	declines day 1 & thereafter
o	20	declines day 2 & thereafter
o	21	declines day 2 & thereafter
o	22	declines day 2 & thereafter
o	23	declines day 2 & thereafter
o	24	declines day 1 & thereafter
o	25	declines day 2 & thereafter
o	26	declines day 2 & thereafter

● disappearing ■ appearing o overall decrease □ overall increase

* no overall change

Table 10 continued

Protein	Occurrence
o 27	declines day 1 & thereafter
o 28	declines day 1 & thereafter
o 29	declines day 1 & thereafter
o 30	declines day 4 & thereafter
o 31	declines day 2 & thereafter
● 32	disappears day 1
● 33	disappears day 1
o 34	declines day 4 & thereafter
o 35	declines day 4 & thereafter
o 36	declines day 1 & thereafter
o 37	declines day 1 & thereafter
● 38	declines day 1, disappears day 5
o 39	declines day 2 & thereafter
o 40	declines day 2 & thereafter
o 41	declines day 2 & thereafter
o 42	declines day 2 & thereafter
o 43	declines day 2 & thereafter
o 44	declines day 2 & thereafter
o 45	declines day 2 & thereafter
● 46	declines day 3 & thereafter, disappears day 7
● 47	declines day 3 & thereafter, disappears day 7
● 48	declines day 2 & thereafter, disappears day 5
● 49	declines day 3 & thereafter, disappears day 7
□ 50	increases day 5 & thereafter
o 51	declines day 3 & thereafter
□ 52	increases day 1 & thereafter
□ 53	increases day 1 & thereafter

Table 10 continued

Protein	Occurrence
● 54	disappears day 2
● 55	declines day 2, disappears day 7
● 56	declines day 1, disappears day 7
□ 57	appears day 4, thereafter increasing
□ 58	increases day 5 & thereafter
■ 59	appears day 4, thereafter increasing
□ 60	increases day 4 & thereafter
● 61	increases day 1 & thereafter, disappears day 3
○ 62	declines day 2 & thereafter

However 7 proteins increase in abundance relative to the general pattern, 1 of which appears de novo (59) after 4 days in ABA.

Although DNA synthesis is inhibited before protein synthesis as a whole, there is no reason to suppose that the synthesis of certain proteins, perhaps those involved in cell division, might not be selectively altered by ABA within hours of ABA application. As discussed in section 4.10 however, ABA could have many sites of action.

The effect of ABA on RNA metabolism in general was far less striking than either DNA or protein synthesis, and a general inhibition of transcription would not result in the enhanced synthesis of certain proteins as has been found. In addition, the inhibition of protein synthesis always preceded that of RNA synthesis. Certainly the effects of ABA on the major RNA species appeared to be of little consequence to Spirodela in the production of turions, as was shown by inhibitor studies (Stewart and Smith, 1972), and is more likely to be the result rather than the cause of growth inhibition.

The de novo production of specific mRNAs and inhibition of the production of others could however account for the protein changes found during turion formation. In order to examine whether the selective in vivo changes in the polypeptide pattern of the developing turion were paralleled by changes in the translatable RNA content at each developmental stage, RNA from such stages were translated using the wheat-germ cell free protein synthesising system.

5.2 IN VITRO CHANGES IN TRANSLATABLE RNA DURING TURION FORMATION

5.2.1 Extraction and Purification

Nucleic acid was extracted from developing turions by the method of Leaver and Ingle (1971), and DNA and low molecular weight RNA removed with LiCl (Palmiter, 1974). Other methods of extraction and purification were investigated since the purity of the RNA obtained was not as high as was hoped, probably due to contaminating polyphenols.

RNA extracted in phenol/chloroform (Higgins et al., 1976) gave an OD_{260}/OD_{280} ratio of 1·56. When the RNA obtained by this method was translated using the wheat-germ system, no discrete products were seen on SDS polyacrylamide gels (Fig. 61b). However when phenol/cresol was used for the extraction method, the RNA appeared totally undegraded and translated well in the wheat-germ system, as long as the RNA was precipitated by ethanol in the presence of 0·1 M sodium acetate pH 5·0 (Fig. 61a). Of the various methods used to further purify the RNA after ethanol precipitation of the nucleic acids, LiCl was preferred, as this treatment increased the translational capacity of the RNA, without any lowering of the $OD_{260/280}$ ratio. Precipitation of RNA by cetyltrimethylammonium bromide, alone (Higgins et al., 1976), or in conjunction with 2-methoxyethanol (Ralph and Bellamy, 1964), also increased the translational capacity of the RNA, but this procedure resulted in low recovery of RNA. No significant differences between purification treatments could be detected in the ability of the RNA to be translated into high molecular weight products (Fig. 61).

Figure 61

The effect of extraction and purification methods on the translational capacity of RNA from S. polyrrhiza (N). Translational capacity was determined in the wheat-germ assay. Wheatgerm products are shown above and the nucleic acid which was also run on 2·4% polyacrylamide gels is shown below.

a) Phenol/cresol;154,645 cpm;(3.2% incorporated)

b) Phenol/chloroform;123,215 cpm;(2·5% incorporated)

c) Phenol/cresol + LiCl;131,145 cpm;(2·7% incorporated)

d) Phenol/cresol + LiCl;194,950 cpm;(3·7% incorporated)

e) Phenol/cresol + cet. bromide;223,460 cpm;(4·3% incorporated)

f) Phenol/cresol + cet. bromide + MO;188,640 cpm;(3·6% incorporated)

---

a) Phenol/cresol;10 μg RNA; R = 1·56

d) Phenol/cresol + LiCl; 10 μg RNA; R = 1·71

e) Phenol/cresol + cet. bromide; 10 μg RNA; R = 1·60

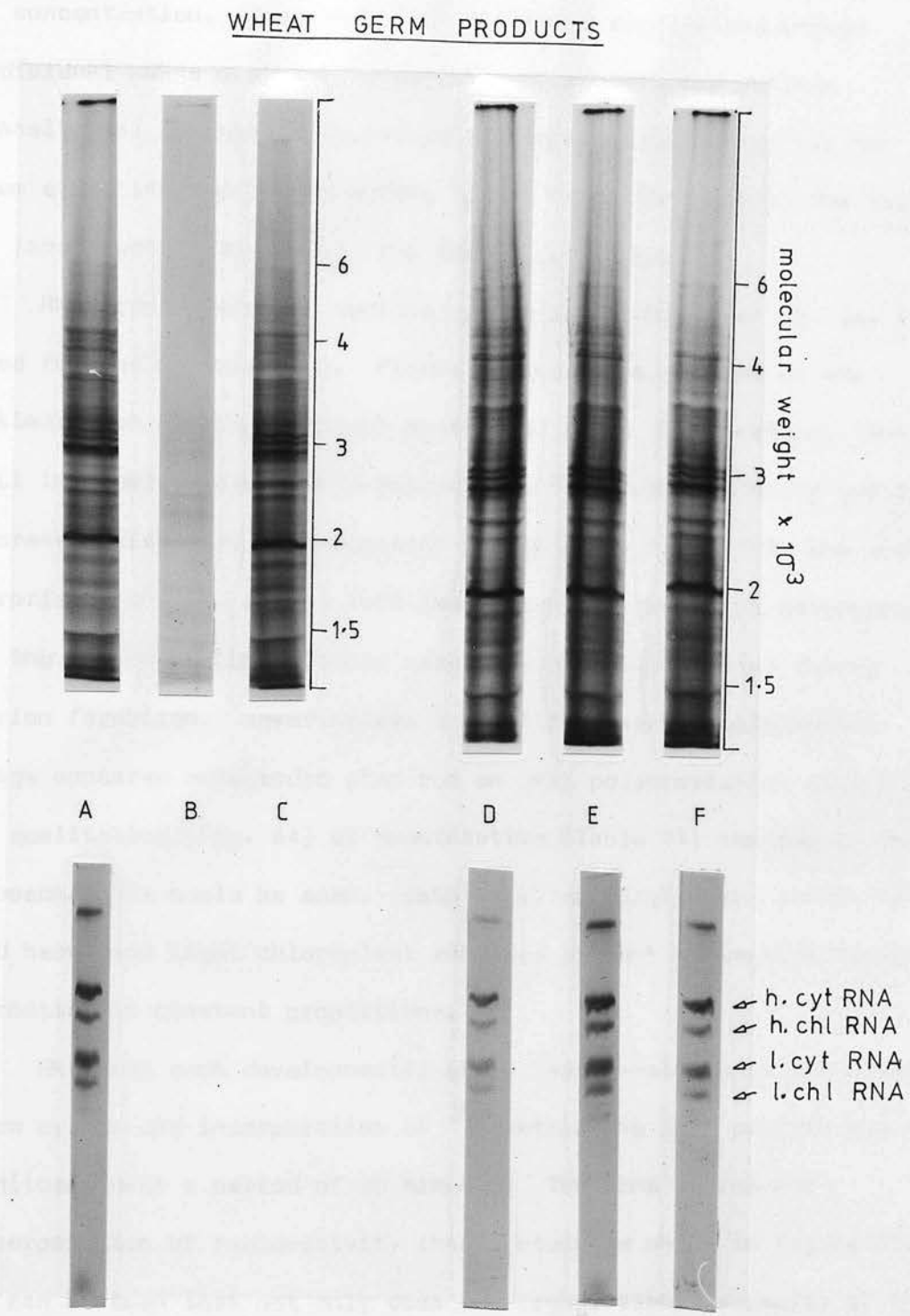
f) Phenol/cresol + cet. bromide + MO; 10 μg RNA; R = 2·04

$R = \frac{OD_{260}}{OD_{280}}$

h = heavy l = light cyt = cytoplasmic

chl = chloroplast

FIGURE 61


WHEAT GERM PRODUCTS
6
4
3
2
1·5
molecular weight x 10^{-3}
A
B
C
D
E
F
h. cyt RNA
h. chl RNA
l. cyt RNA
l. chl RNA
RNA GELS

5.2.2 Optimisation of the Wheat-germ System for S. polyrrhiza RNA

The wheat-germ system was optimised for Spirodela nucleic acid with respect to nucleic acid concentration, Mg^{2+} concentration and K^{+} concentration, since mRNA from different species and indeed individual mRNAs may have different requirements for optimum translation. It was not possible to optimise the system for RNA from each different stage during turion formation, due to the lack of large quantities of RNA from the latter stages.

RNA from developing turions before the addition of ABA was thus used for the optimisation. Figure 62 shows the results of the optimisation of the wheatgerm system for these 3 parameters. The LiCl insoluble nucleic acid extractable from the developing turions decreased with time of incubation in ABA (Fig. 63). This was rather surprising in view of the fact that total RNA levels as determined by the method of Guinn (1966) were only slightly lowered during turion formation. Nevertheless the RNA from each developmental stage appeared undegraded when run on 2·4% polyacrylamide gels and no qualitative (Fig. 64) or quantitative (Table 11) changes in the ribosomal RNA could be seen. Both heavy and light cytoplasmic rRNA, and heavy and light chloroplast rRNA was present throughout turion formation in constant proportions.

RNA from each developmental stage was translated in the wheat-germ system and incorporation of ^{35}S methionine into protein was monitored over a period of 90 minutes. The time course for incorporation of radioactivity into protein is shown in figure 65. It can be seen that not only does the translational capacity of the RNA decrease during turion formation, but this effect is of course

Figure 62

Optimisation of the wheat-germ system for S. polyrrhiza (N) RNA. Effect of K^+, Mg^{2+} and nucleic acid concentration on in vitro translation. Wheatgerm in vitro incorporations were carried out using varying concentrations of K^+ (a) Mg^{2+} (b) and nucleic acid (c). Incorporation was calculated as cpm incorporated into protein $\times 10^3$. 50 μl incubation^{-1}. The normal levels used were 94 mM K^+, 2·00 mM Mg^{2+} and 6 μg nucleic acid. Incorporation was corrected for endogenous activity of the wheat-germ preparation.

FIGURE 62

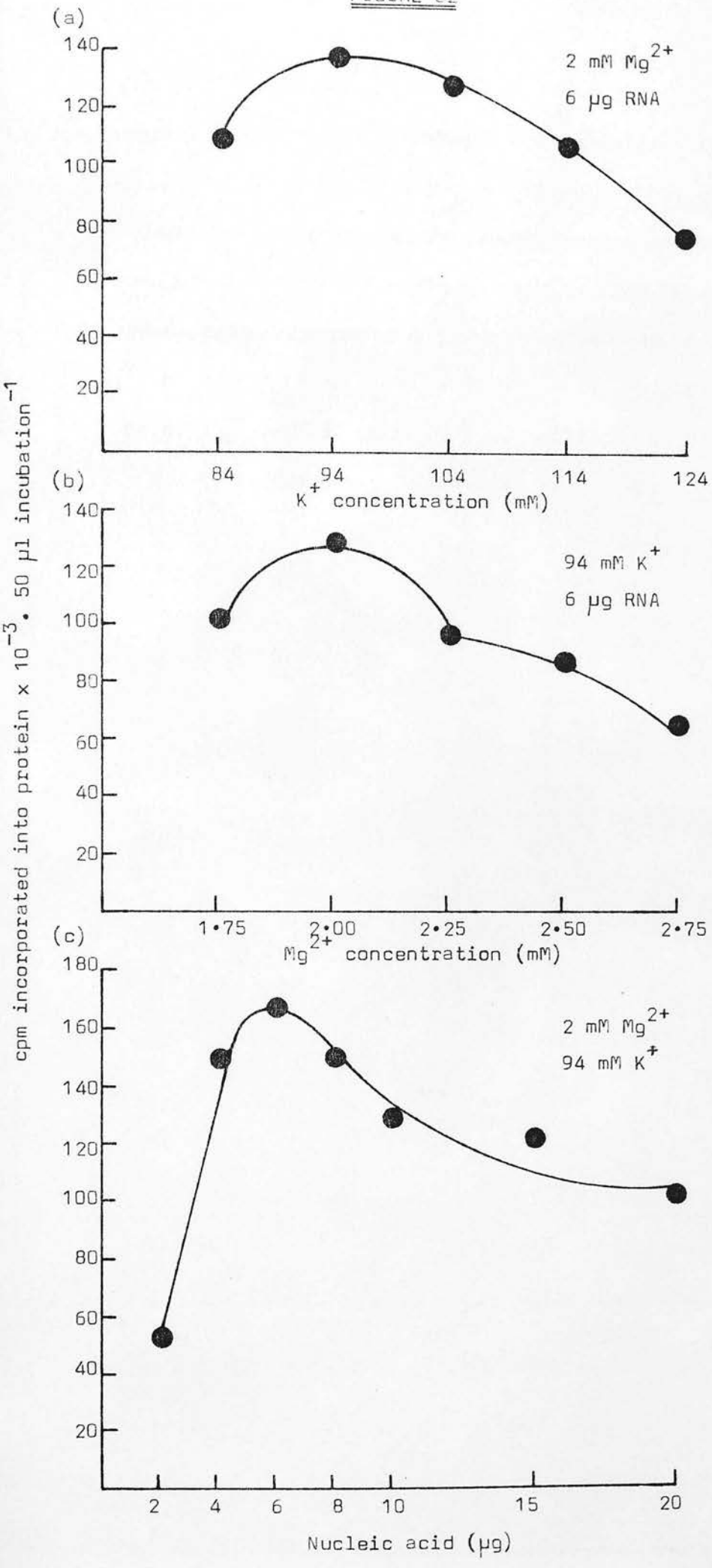

(a)
140
120
100
80
60
40
20
2 mM Mg^{2+}
6 µg RNA
84
94
104
114
124
K^+ concentration (mM)
(b)
140
120
100
80
60
40
20
94 mM K^+
6 µg RNA
1·75
2·00
2·25
2·50
2·75
Mg^{2+} concentration (mM)
(c)
180
160
140
120
100
80
60
40
20
2 mM Mg^{2+}
94 mM K^+
2
4
6
8
10
15
20
Nucleic acid (µg)
cpm incorporated into protein x 10^{-3} • 50 µl incubation^{-1}

Figure 63

The level of LiCl insoluble extractable nucleic acid during turion development of S. polyrrhiza (N) in 1 x 10^{-7} M ABA. Nucleic acid was extracted from each developmental stage, purified with LiCl and nucleic acid was determined spectrophotometrically. The nucleic acid extractable from each frond could then be calculated.

FIGURE 63

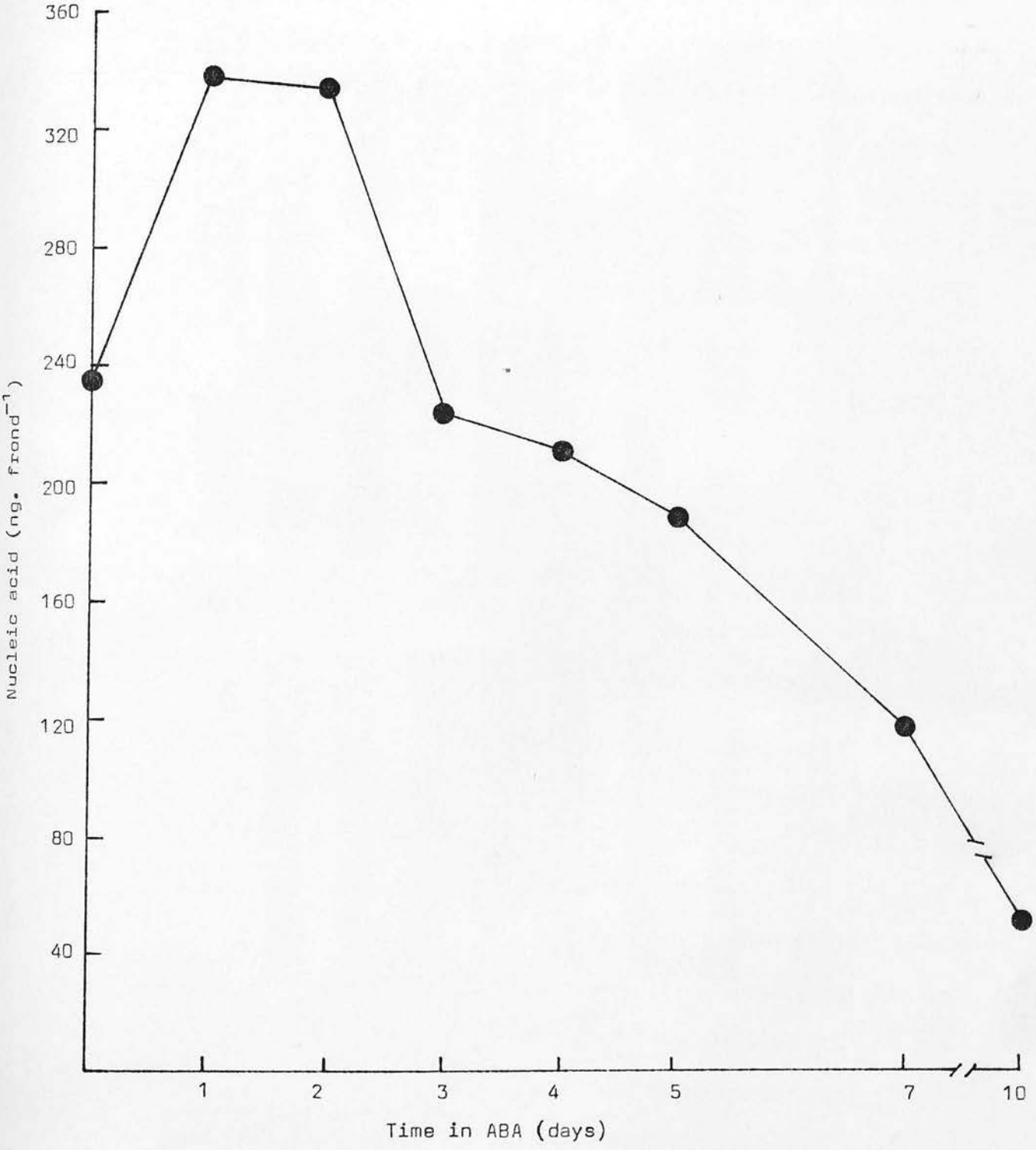

360
320
280
240
200
160
120
80
40
Nucleic acid (ng. frond^{-1})
1
2
3
4
5
7
10
Time in ABA (days)

Figure 64

In vitro translation of RNA during the development of the turion of S. polyrrhiza (N) in 1 x 10^{-7} M ABA. LiCl insoluble phenol/cresol extractable nucleic acid from each developmental stage was translated in the wheat-germ system. The products after 90 min incorporation were run on polyacrylamide gels. The labelled products were visualised by fluorography.

a) no RNA 18,000 cpm
b) day 0 RNA 120,000 cpm
c) day 1 RNA 102,000 cpm
d) day 2 RNA 85,000 cpm
e) day 3 RNA 77,000 cpm
f) day 4 RNA 75,000 cpm
g) day 5 RNA 51,000 cpm
h) day 7 RNA 25,000 cpm
i) day 10 RNA 22,000 cpm
fully developed turions

Equal amounts of RNA (6 µg) were used in the incuabtions.

The nucleic acid extractable at each stage was also run on 2·4% polyacrylamide gels to check the integrity of the RNA. 5 main bands are observed on the gels each characterised by a peak number. The ethidium bromide gels were then scanned in order to detect any differences between the ratios of the major ribosomal RNA peaks.

Peak 2 = light chloroplast rRNA

Peak 3 = light cytoplasmic rRNA

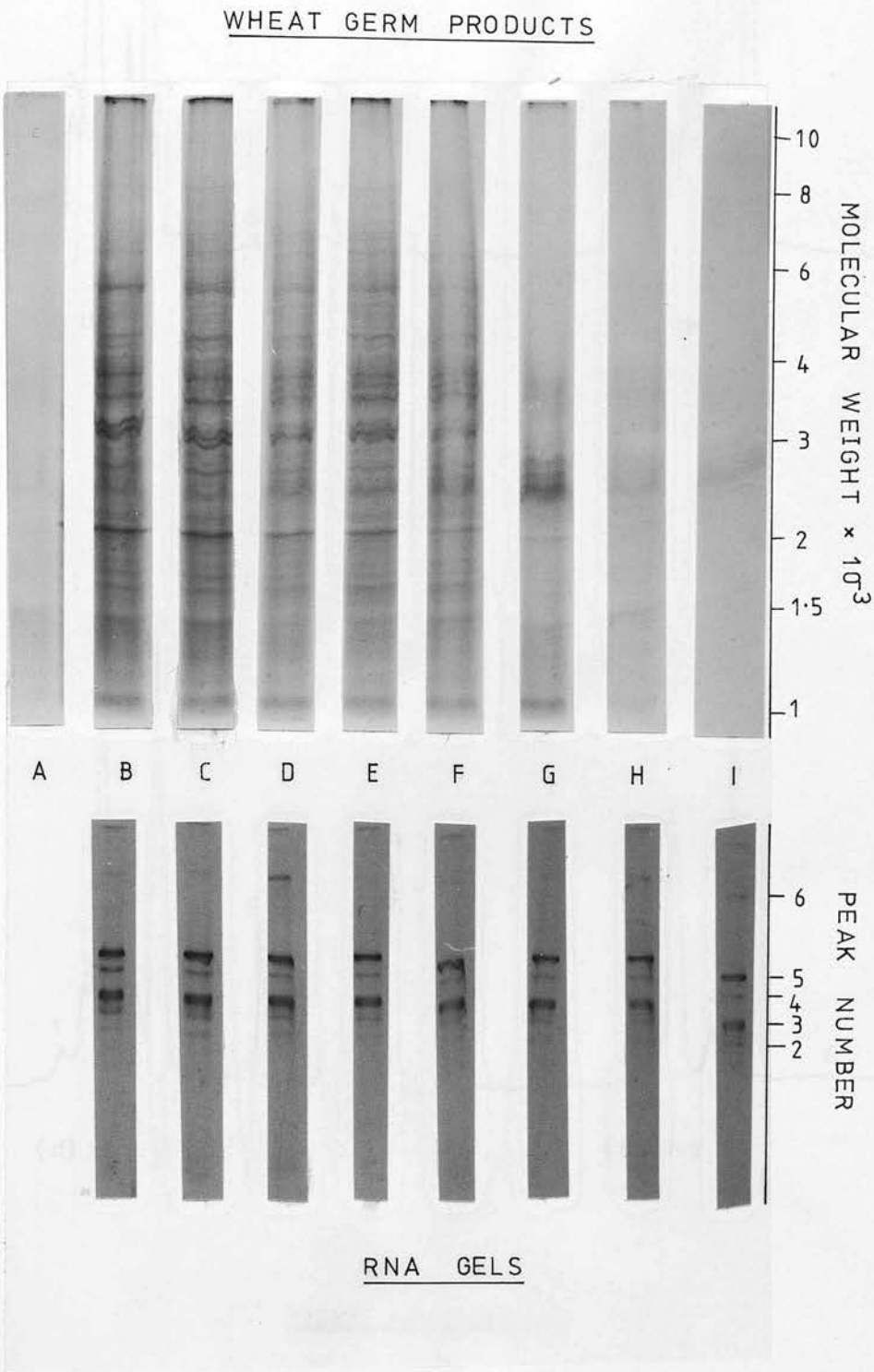
Peak 4 = heavy chloroplast rRNA

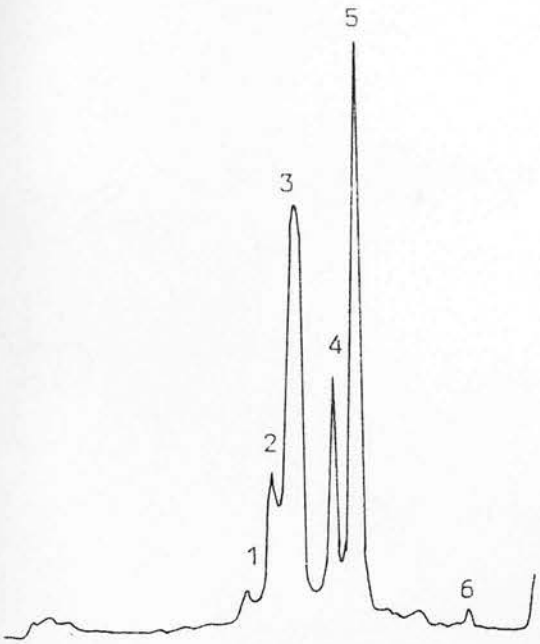
Peak 5 = heavy cytoplasmic rRNA

Peak 6 = probably aggregates of rRNA or DNA (DNA should have been removed by the LiCl treatment)

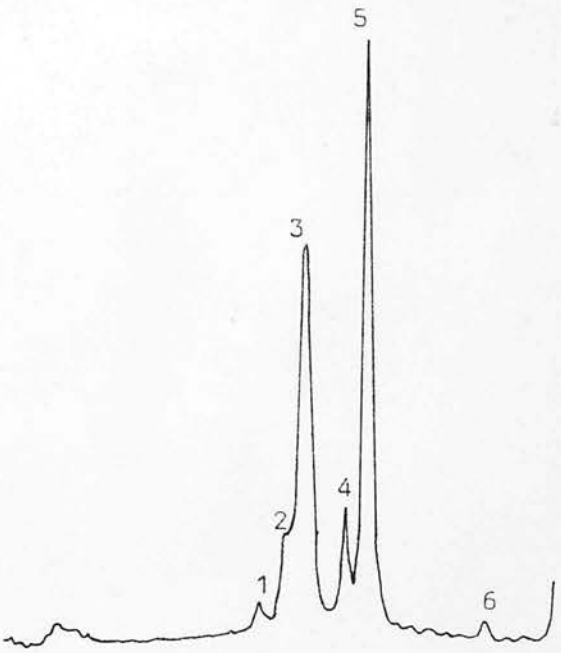
The peak ratios are shown in Table 11.

FIGURE 64

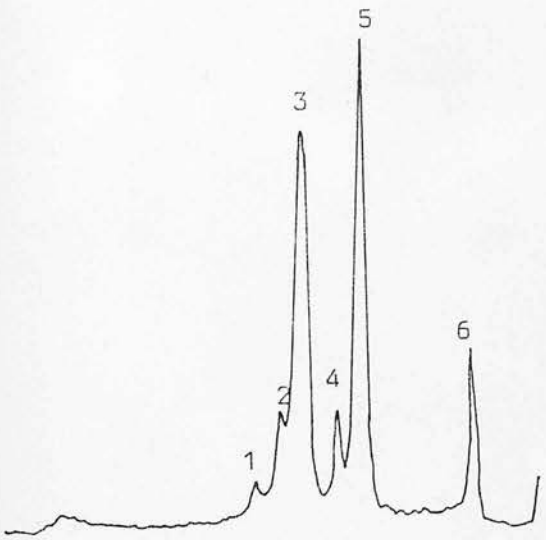

WHEAT GERM PRODUCTS
10
8
6
4
3
2
1·5
1
MOLECULAR WEIGHT × 10⁻³
A
B
C
D
E
F
G
H
I
6
5
4
3
2
PEAK NUMBER
RNA GELS



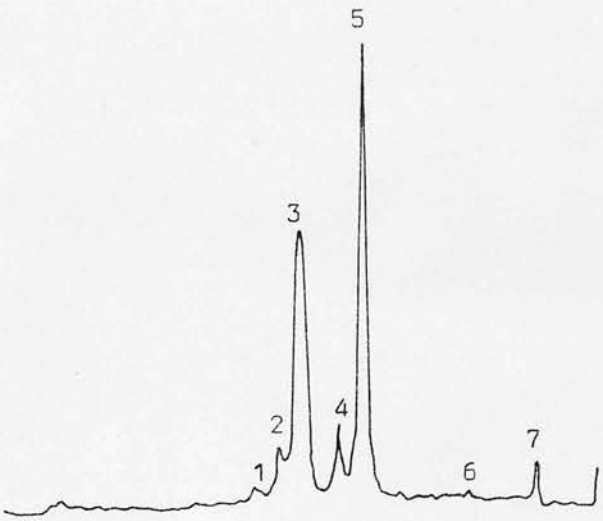
(b) day 0



(c) day 1



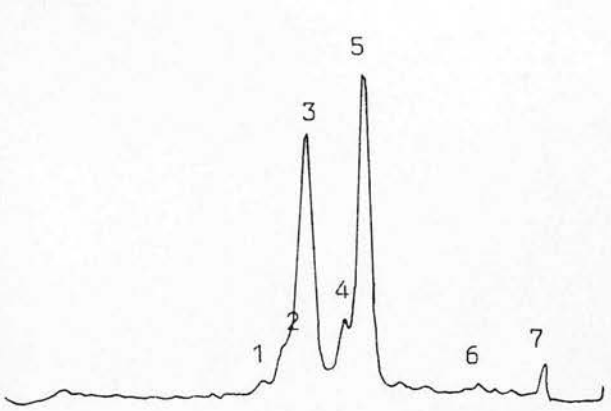
(d) day 2



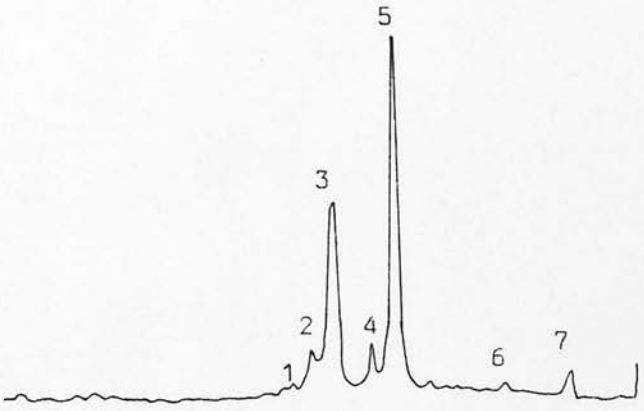
(e) day 3

FIGURE 64 continued

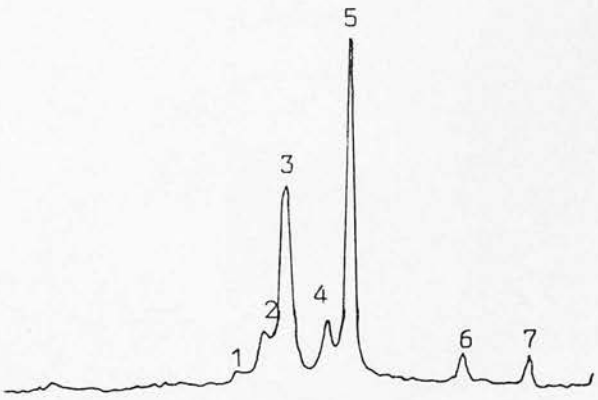
FIGURE 64 continued



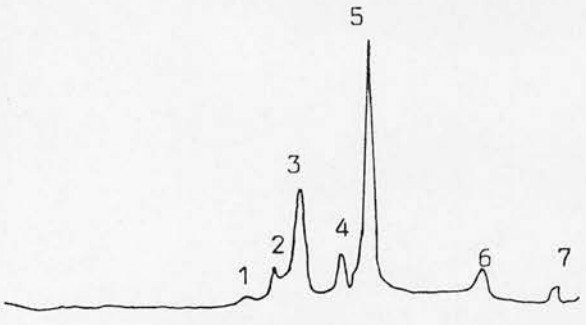
(f) day 4



(g) day 5



(h) day 7



(i) day 10

Table 11: The peak ratios of rRNA during the development of the turion of S. polyrrhiza (N).

Day	Areas under the peaks (arbitrary units)			
	Peak 2	Peak 3	Peak 4	Peak 5
0	44	117	55	120
1	44	146	57	165
2	41	150	48	135
3	30	120	46	130
4	27	96	34	113
5	29	95	36	112
7	32	84	37	86
10	27	84	39	98

The peaks referred to are to be found in Figure 64

Day	Peak ratios			
	P5/P3	P5/P4	P3/P2	P4/P2
0	1•03	2•18	2•66	1•25
1	1•13	2•89	3•32	1•30
2	0•90	2•81	3•66	1•17
3	1•08	2•83	4•00	1•53
4	1•18	3•32	3•56	1•26
5	1•18	3•11	3•28	1•24
7	1•02	2•32	2•63	1•16
10	1•17	2•51	3•11	1•44

There appear to be no qualitative or quantitative differences in the distribution of the cytoplasmic or chloroplast rRNAs.

10 µg of nucleic acid applied to each gel in Figure 64.

Figure 65

Time course of *in vitro* incorporation of ^{35}S methionine into protein by RNA extracted during the development of the turion of *S. polyrrhiza* (N) in 1 x 10^{-7}M ABA.

+ no RNA

● day 0 RNA

o day 1 RNA

■ day 2 RNA

□ day 3 RNA

▲ day 4 RNA

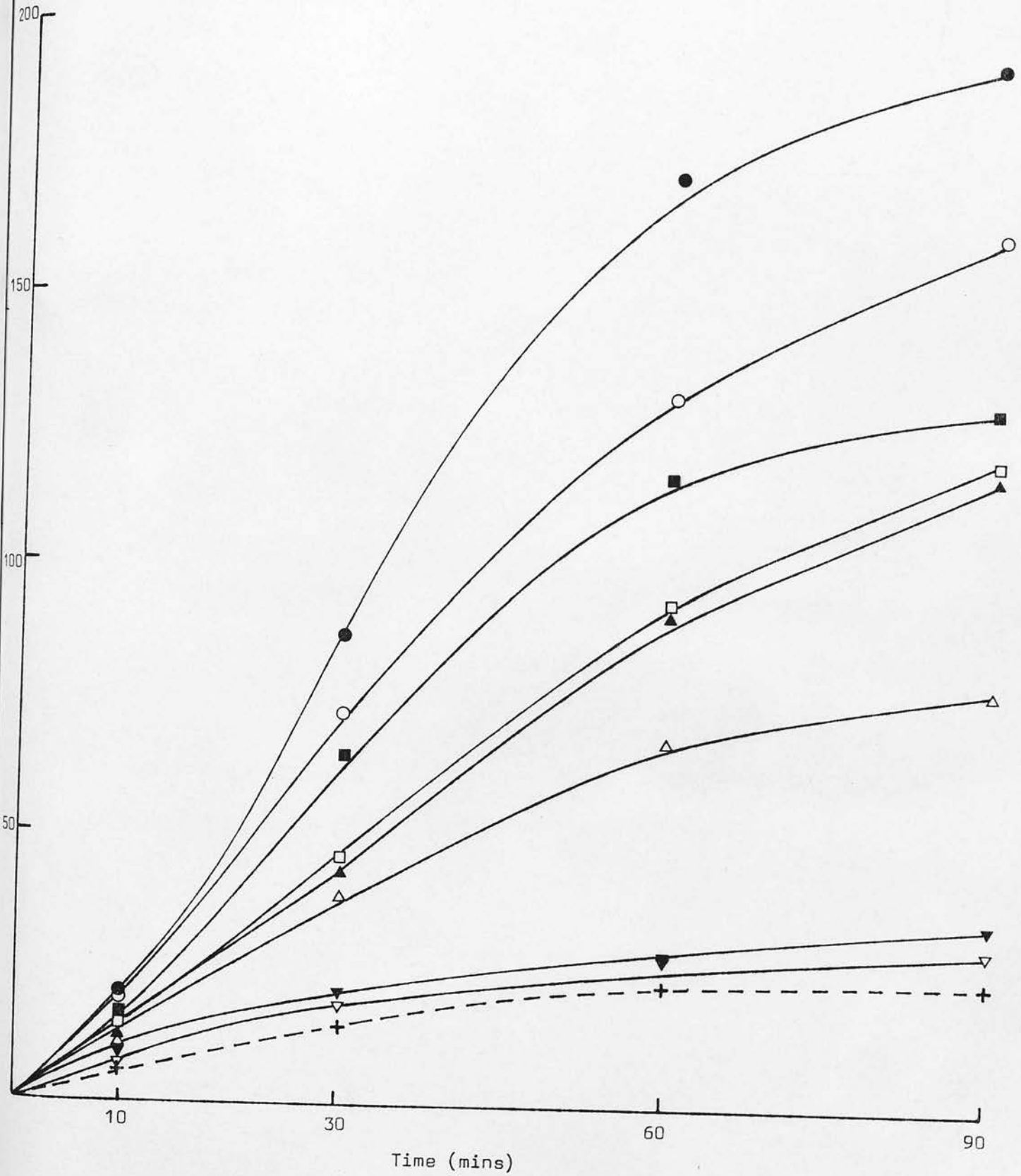
△ day 5 RNA

▼ day 7 RNA

▽ day 10 RNA (fully mature turion)

Equal amounts of nucleic acid were added to each incorporation (6 µg). Incorporations were corrected for radioactivity not incorporated into protein.

FIGURE 65



magnified since there was also a decrease in the amount of LiCl insoluble RNA extractable as turion formation proceeded. Indeed the RNA extracted from the fully mature turion had no capacity for translation.

It is tempting to speculate that the reduced rate of protein synthesis found *in vivo* in the developing turion may be due, at least in part, to a decrease in the availability of mRNA. Whether this decrease arises from decreased transcription, decreased precursor RNA processing or an increase in the rate of degradation of mRNA is unknown. Another possibility that cannot be ignored is that during extraction of RNA from the developing turions, RNAase activity may have increased during turion formation, thus rendering the mRNA inactive. Likewise the extraction procedure might release inhibitors of translation, perhaps present in higher concentration in the more mature turions, that would be compartmentalised *in vivo*.

Some mRNAs must be present in the turion, since *in vivo* many polypeptides are labelled, although their synthesis is very much reduced compared to the early stages in turion formation. The mRNAs coding for these proteins may, as has been pointed out above, have been degraded, or the translation requirements may have differed for the mRNA extracted at this stage.

More interesting perhaps, are the patterns of *in vitro* labelled polypeptides produced by running the products of the translation on 2-dimensional polyacrylamide gels (Fig. 66). Within 1 day of ABA application one prominent protein decreases in abundance (which may be soluble protein 8) while other proteins appear or increase their label relative to the overall pattern. It is virtually impossible

Figure 66

In vitro translation of RNA during the development of the turion of *S. polyrrhiza* (N) in 1 x 10^{-7}M ABA. LiCl insoluble phenol/cresol extractable nucleic acid from each developmental stage was translated in the wheat-germ system. The products after 90 min incorporation were run on 2-dimensional polyacrylamide gels. The labelled products were visualised by fluorography. Equal amounts of incubate were applied to each gel. Each incubate contained 6 µg of nucleic acid.

a) day 0 RNA (untreated) 120,000 cpm

b) day 1 RNA 102,000 cpm

c) day 2 RNA 85,000 cpm

d) day 3 RNA 77,000 cpm

e) day 4 RNA 75,000 cpm

f) day 5 RNA 51,000 cpm

g) day 7 RNA 25,000 cpm

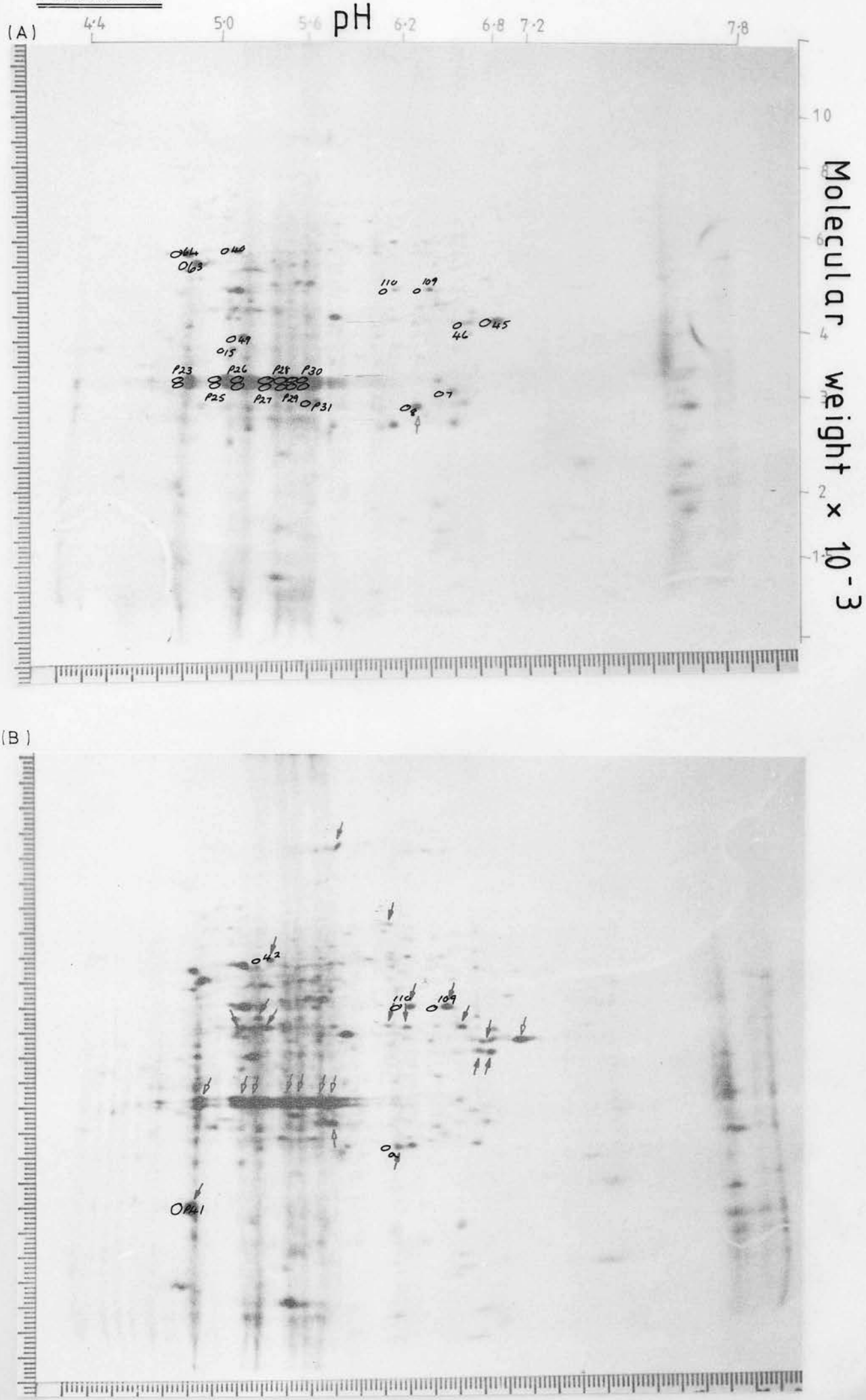
h) day 10 RNA 22,000 cpm

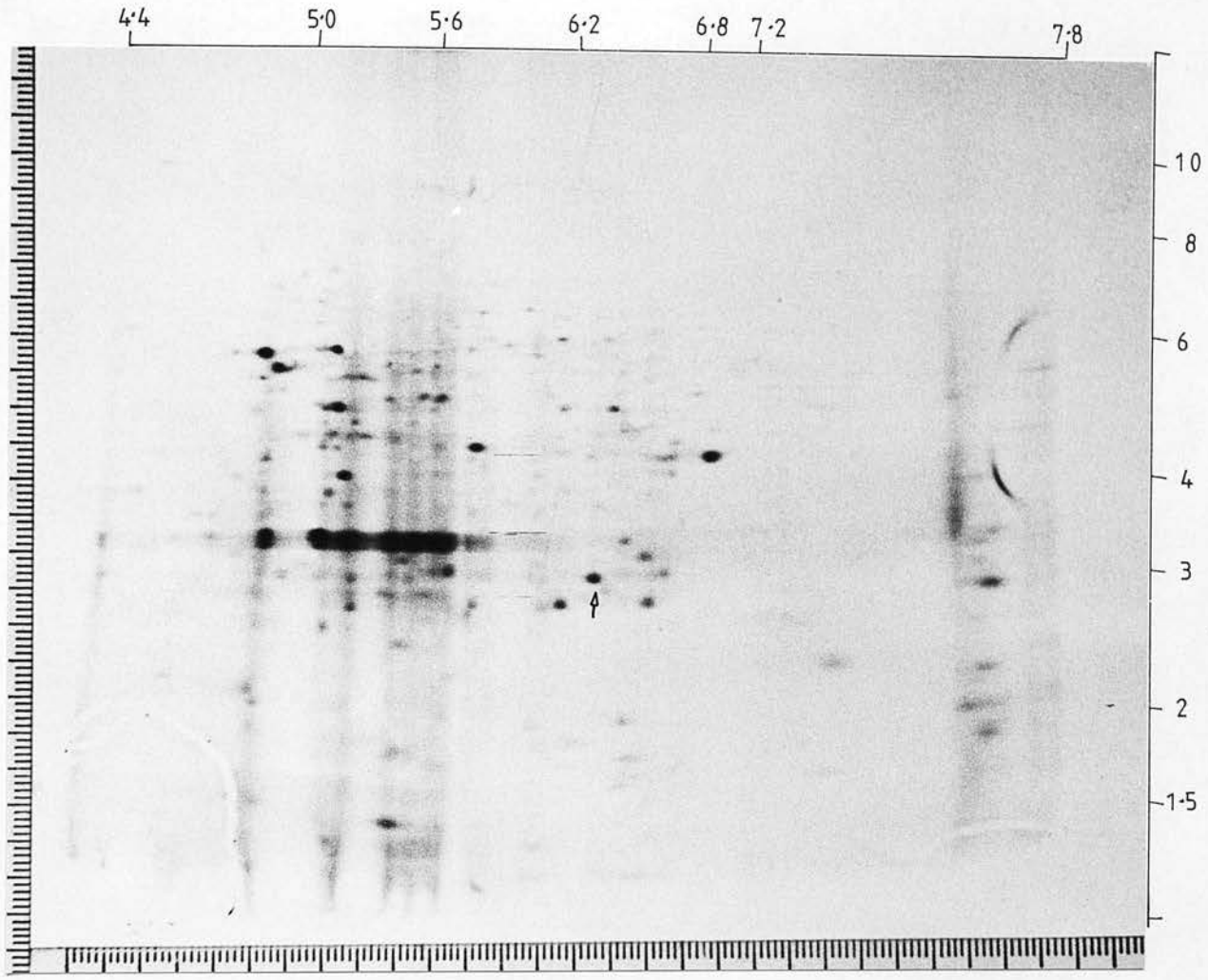
i) no RNA 18,000 cpm

↙ indicates that the product increases from the day before

↗ indicates that the product decreases on the next day

FIGURE 66


(A)
4·4
5·0
5·6
pH
6·2
6·8
7·2
7·8
10
6
4
2
1·5
Molecular weight x 10-3
P23
P25
P26
P27
P28
P29
P30
P31
(B)


4·4
5·0
5·6
6·2
6·8
7·2
7·8
10
8
6
4
3
2
1·5

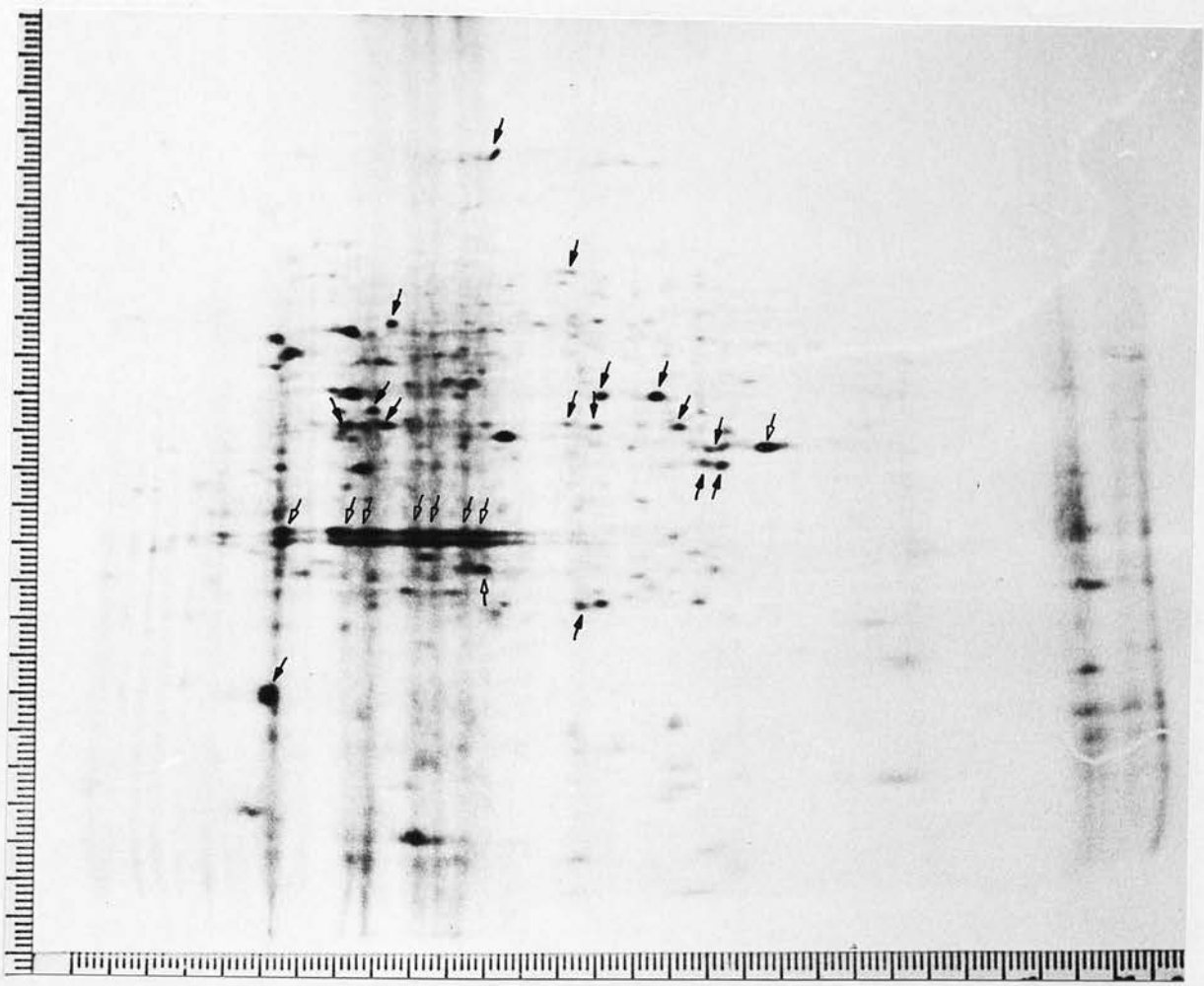
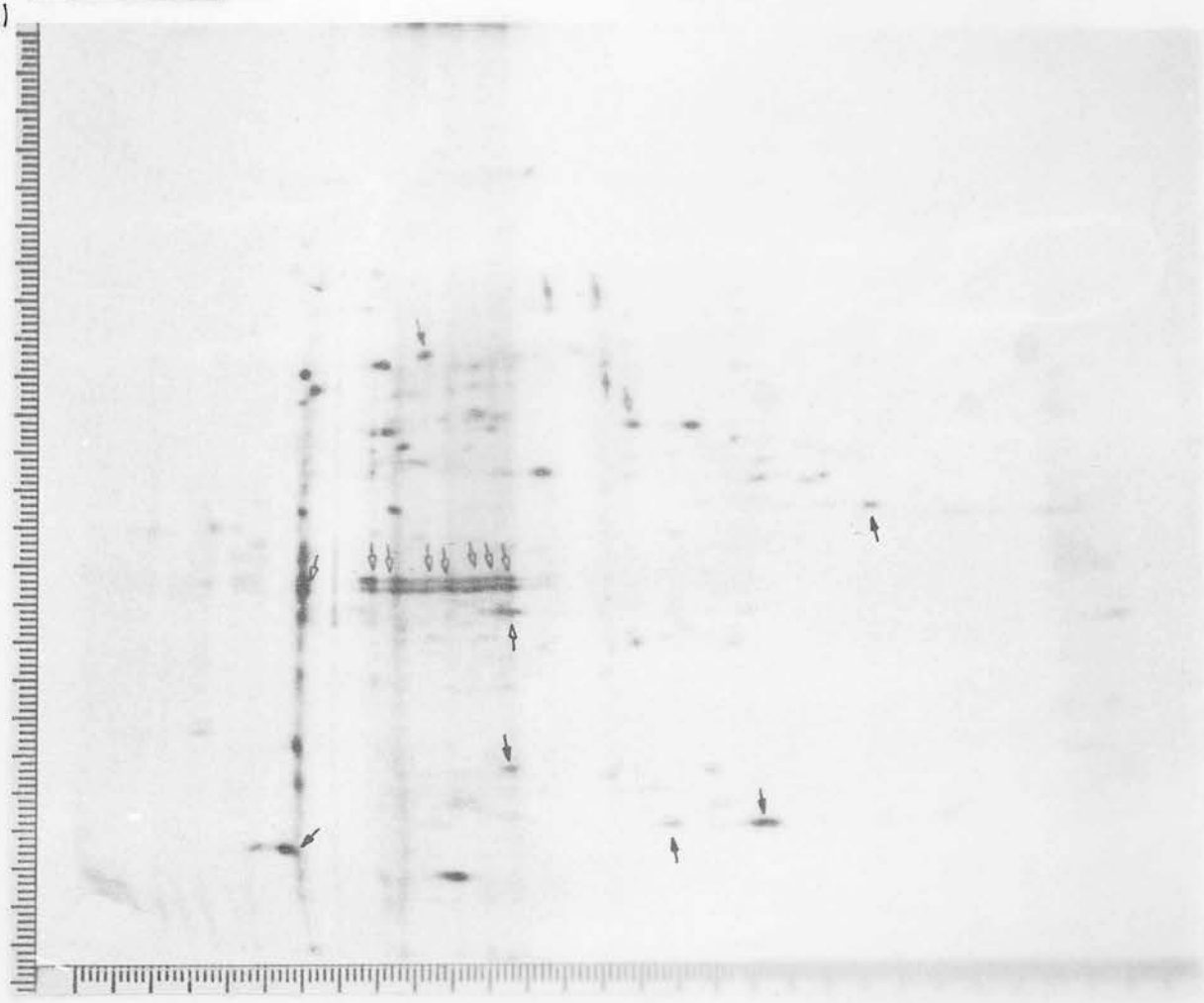
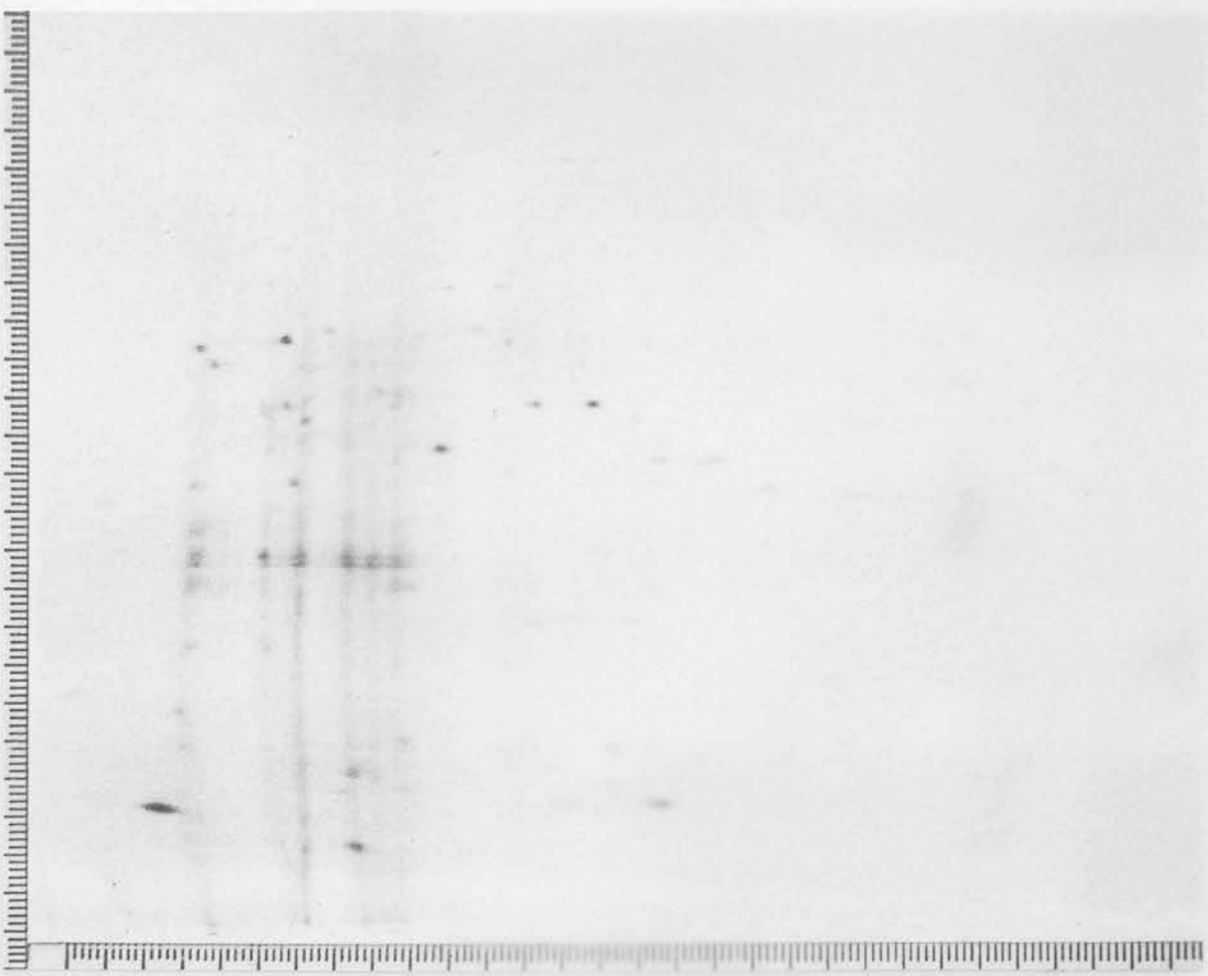


FIGURE 66

(C)



(D)



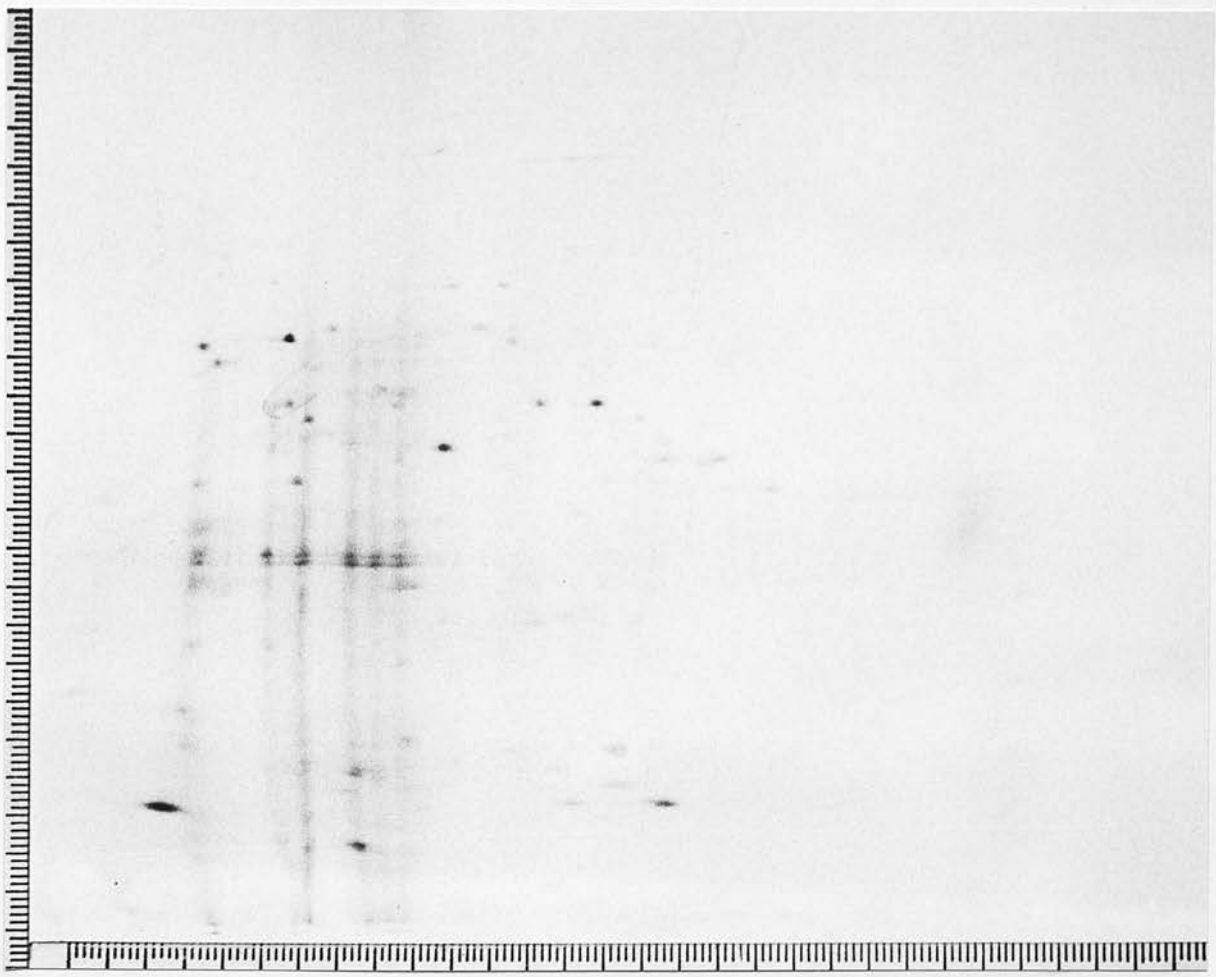
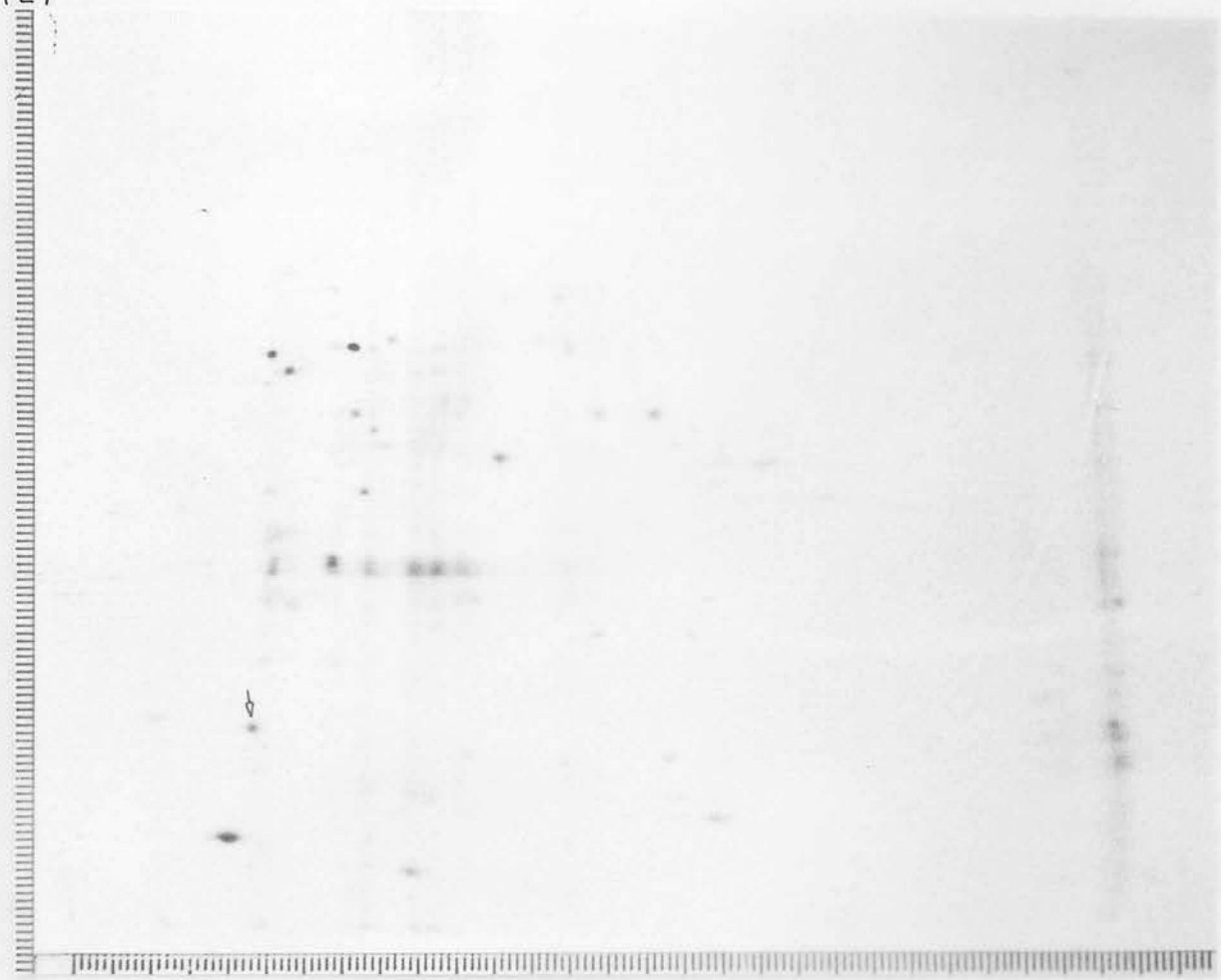
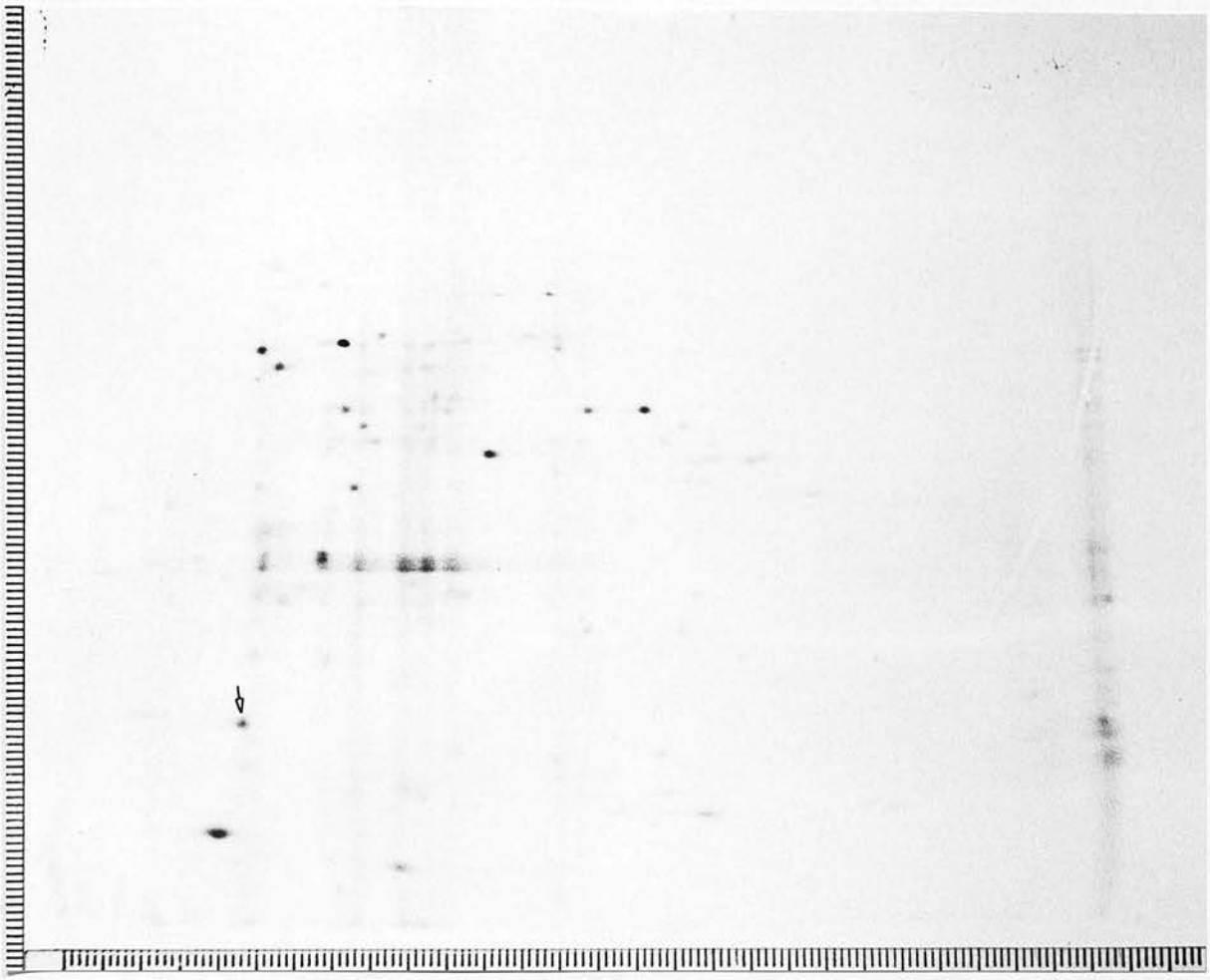


FIGURE 66

(E)



(F)



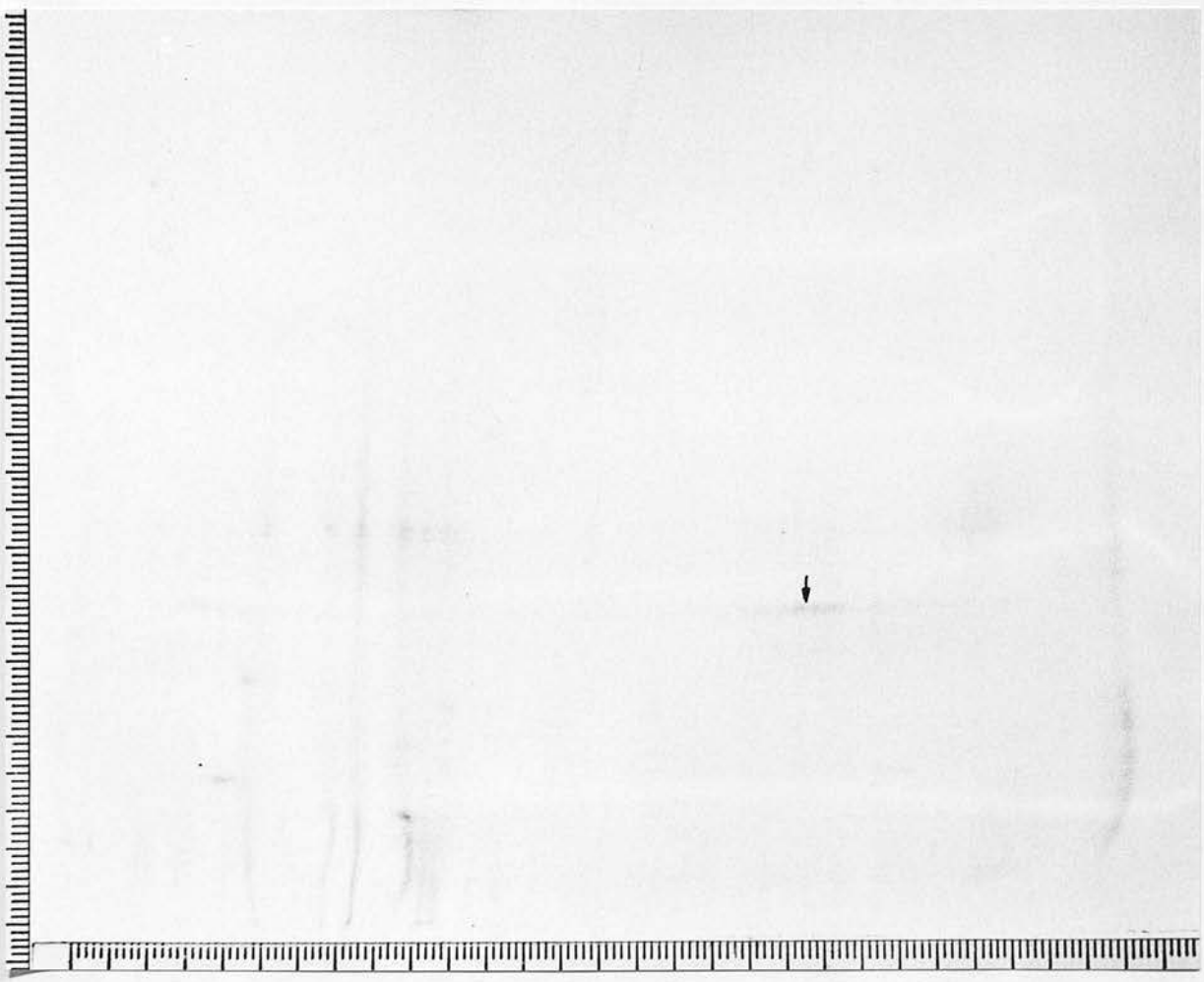
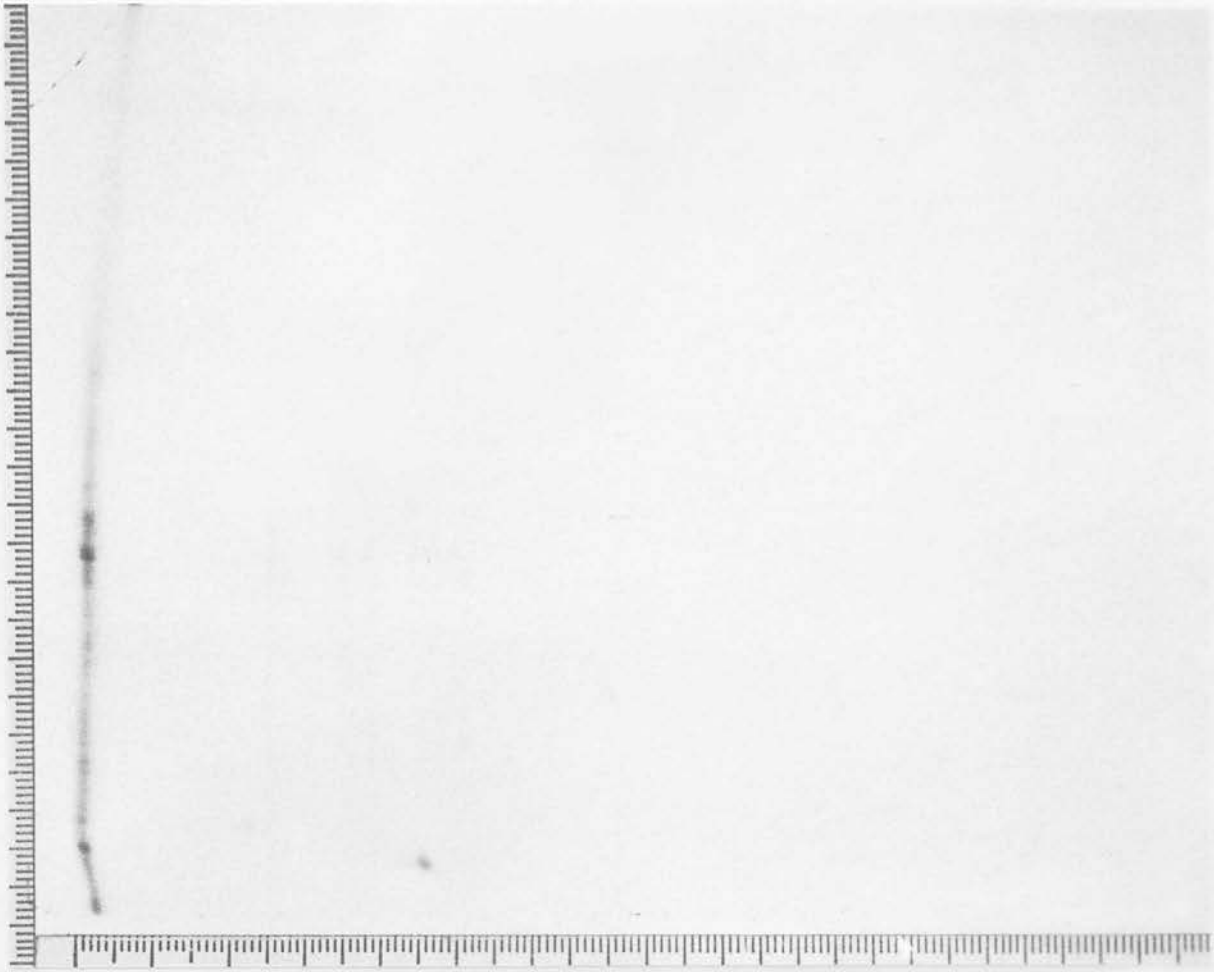
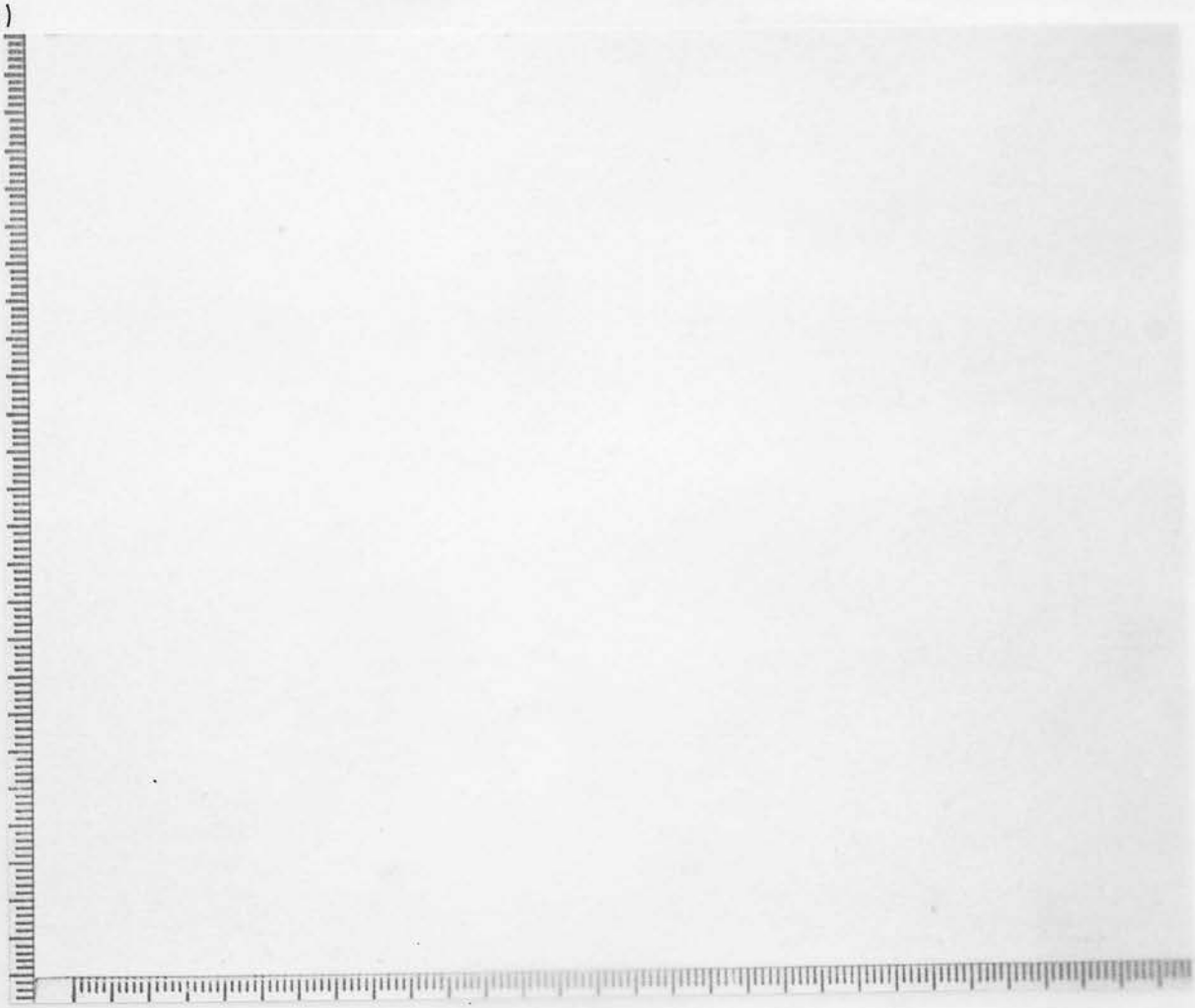


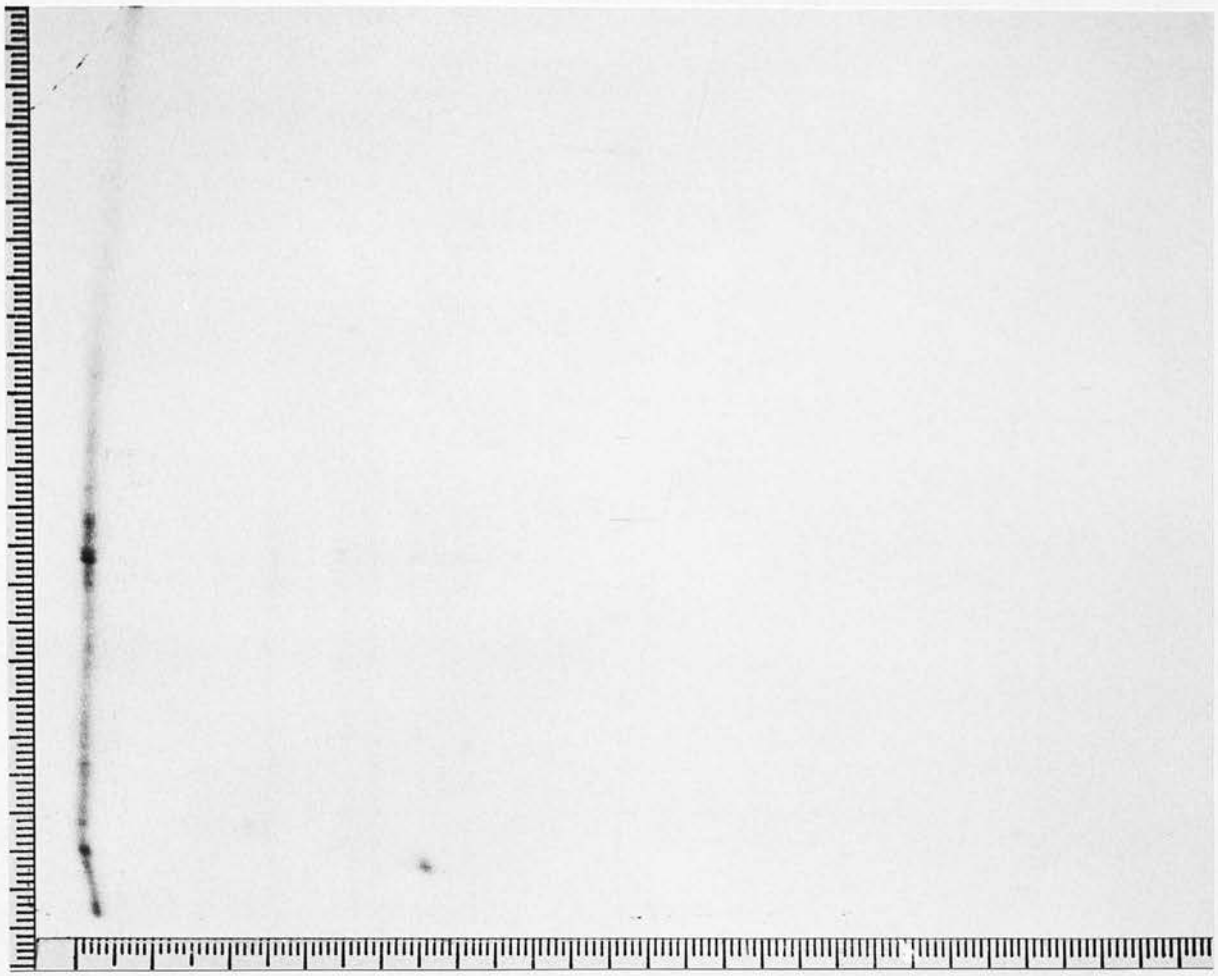
FIGURE 66

(G)



(G)





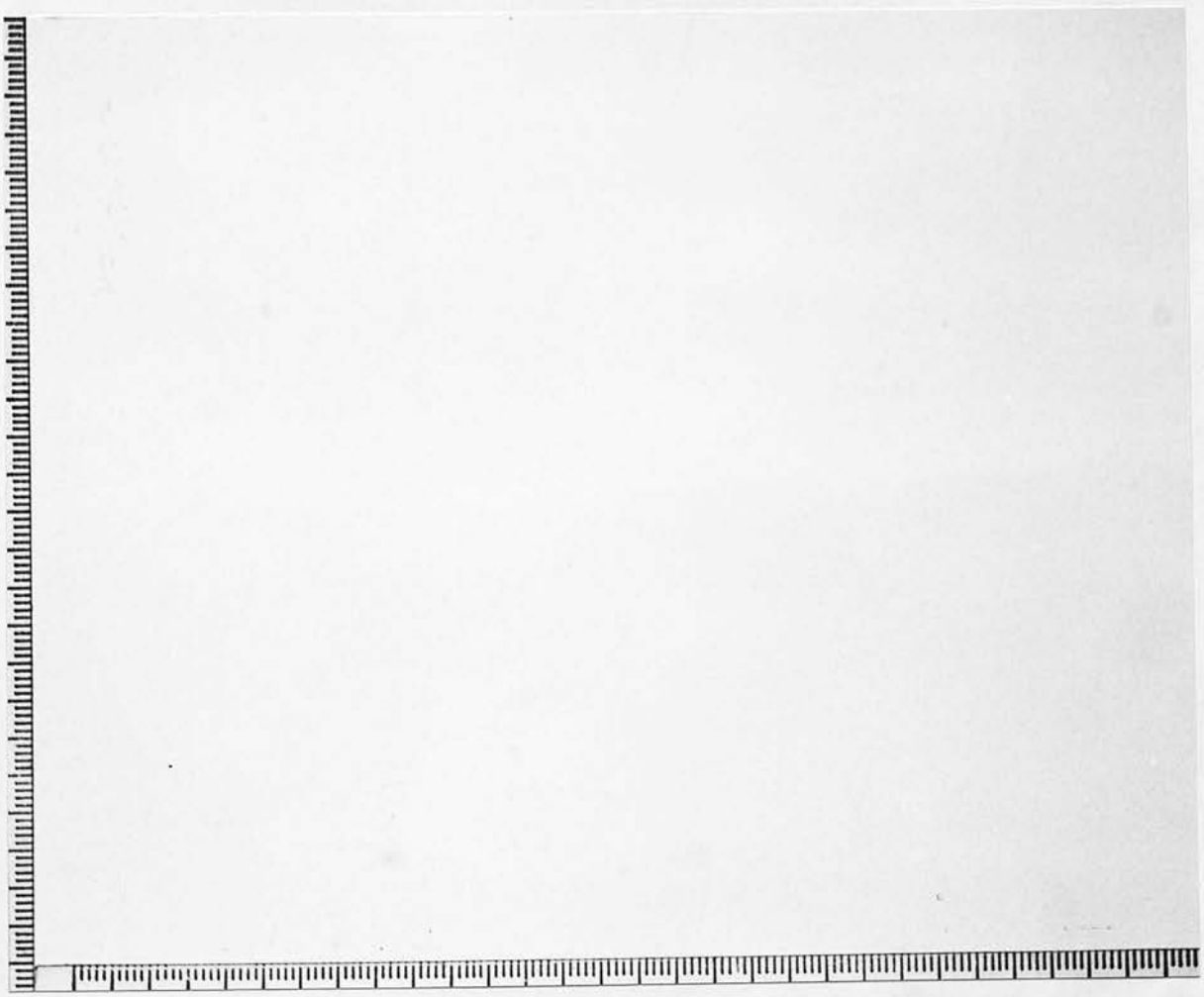
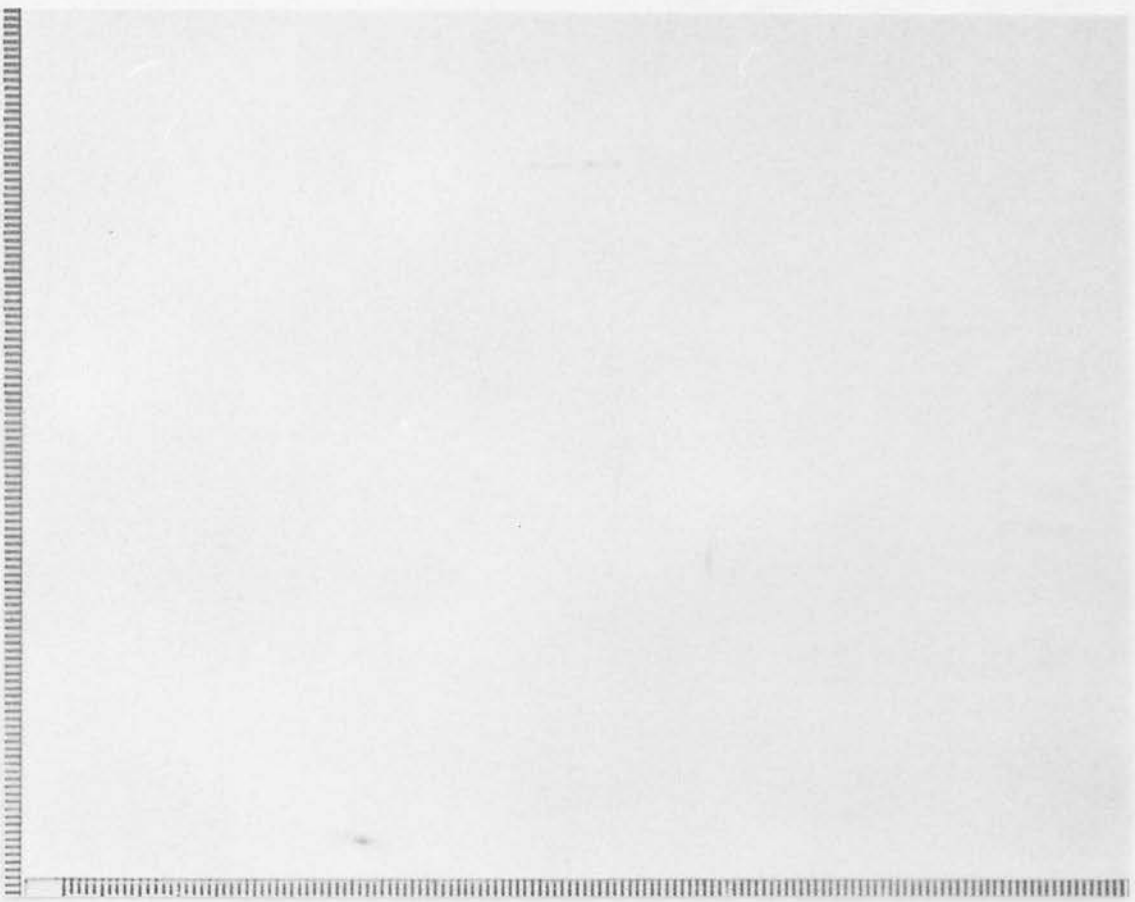
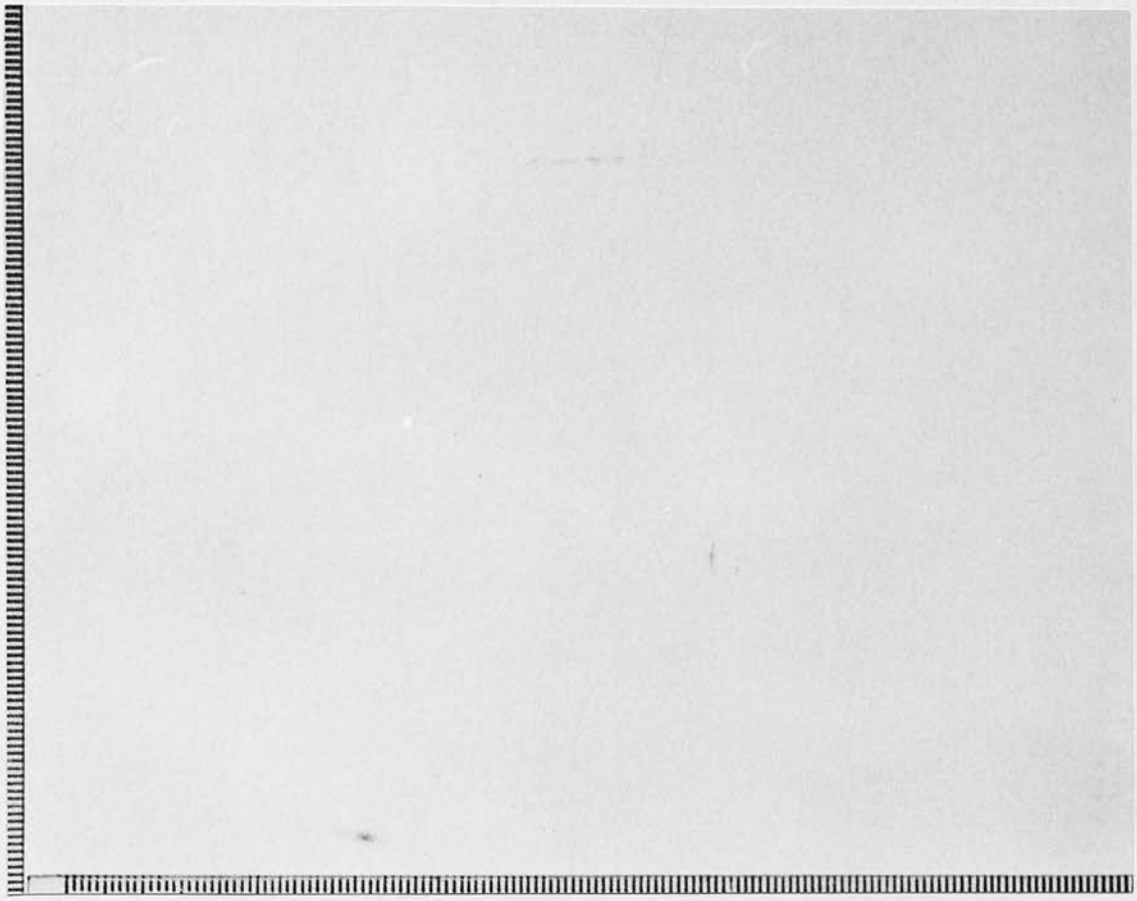


FIGURE 66

(I)





to match the *in vitro* products with those obtained *in vivo*, but the multiple charged polypeptides (P23 - P30) (Fig. 66a) are easily recognisable as membrane proteins (cf. Fig. 60a). These *in vitro* labelled proteins are among the last to disappear as the turion matures.

Of the products that appear on day 1, proteins labelled 42, P41 and (a) are the most prominent. After 2 days in ABA most of the products begin to decrease as the overall label decreases, but once again the inhibition of translation is less pronounced with respect to some products. Indeed after 2 days in ABA, 3 more proteins increase their label relative to the overall pattern (b, c and d). By day 5 however only very few products remain; and in the fully developed turion, there are no products except for the 2 found as a result of the endogenous RNA activity found in the wheat-germ extract.

In order to measure the overall translational capacity of the turion throughout its development, the translational capacity of the aliquots of nucleic acid translated in the wheat-germ system must be multiplied by the total amount of extractable nucleic acid at each developmental stage. The total translatable RNA content during the development of the turion is shown in figure 67.

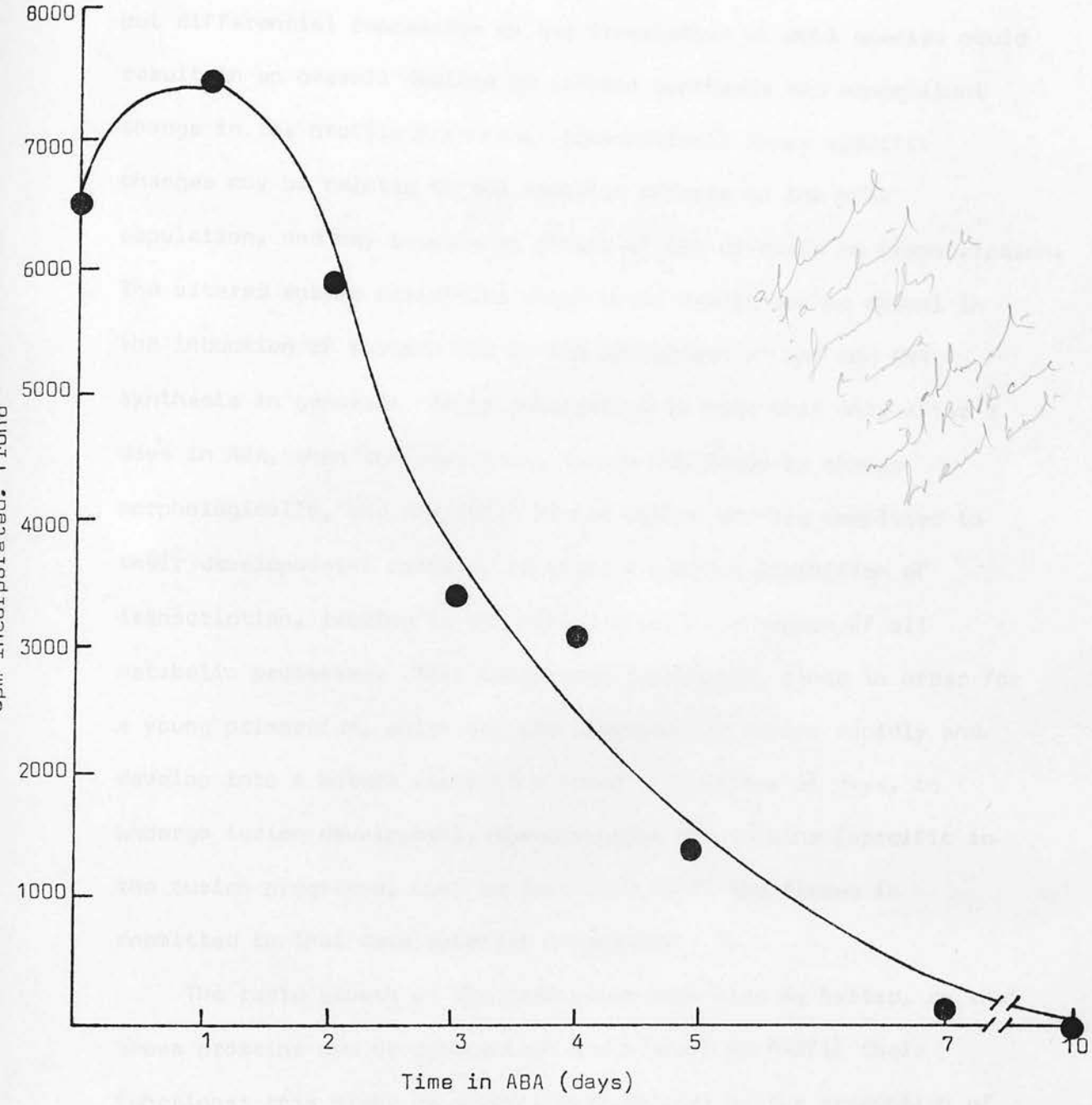
Although protein synthesis was inhibited after only 1 day, the translatable RNA content was not significantly reduced until 3 days after turion induction. It therefore seems unlikely that the large reduction in the availability of messenger RNA is directly responsible for the rapid inhibition of protein synthesis.

Although these experiments were only of a preliminary nature,

Figure 67

The total translatable RNA content during the development of the turion of S. polyrrhiza (N) in 1 x 10^{-7} M ABA. From the cpm incorporated. 50 µl incubation^{-1}. µg RNA^{-1} and the total LiCl insoluble phenol/cresol extractable RNA from each developmental stage, the total level of translatable RNA. frond^{-1} can be calculated.

FIGURE 67


8000
7000
6000
5000
4000
3000
2000
1000
1
2
3
4
5
7
10
Time in ABA (days)

it is conceivable that ABA may induce turion formation initially by interfering with protein synthesis in some direct manner. A general but differential repression in the translation of mRNA species could result in an overall decline in protein synthesis and concomitant change in the profile produced. Alternatively these specific changes may be related to the specific effects on the mRNA population, and may involve an effect of ABA directly on transcription. The altered enzyme activities which would result may be causal in the induction of turions and in the inhibition of DNA and RNA synthesis in general. It is interesting to note that only after 3 days in ABA, when the developing turion has begun to change morphologically, and the cells at the distal end are committed to their developmental pathway, is there a general inhibition of transcription, leading to the almost complete shutdown of all metabolic processes. This would seem reasonable, since in order for a young primordium, which has the potential to expand rapidly and develop into a mature vegetative frond in a matter of days, to undergo turion development, the synthesis of proteins (specific to the turion programme) must be sustained until the tissue is committed to that developmental programme.

The rapid growth of the primordium must also be halted, so that these proteins can be synthesised and allowed to fulfil their functions; this might be accomplished in part by the production of DNA synthesis inhibitors which would slow down the process of cell division, especially in the meristematic pockets of the developing turion. The induction of new proteins necessary for turion formation could be accomplished by transcriptional control i.e. by

the production of new mRNAs, or at a translational or post-translational level i.e. by polypeptide processing, transport or protein turnover.

When these proteins have fulfilled their role and the cell is committed to the turion pathway, both mRNA and rRNA synthesis begin to shut down and the process of dormancy begins. The accumulation of starch and secondary metabolites in the cells of the turion may simply be the result of this shutdown.

The production of turion specific proteins must be quite rapid in order to effect control of DNA synthesis within 3 hours. Control by ABA of the development of the turion at membrane level in concert with control at the molecular level, was a possibility that could not be ignored. For this reason the effect of ABA on ion fluxes in Spirodela polyrrhiza was investigated.

CHAPTER 6

RESULTS AND DISCUSSION

ION TRANSPORT, ABSCISIC ACID AND TURION FORMATION

6.1 ANALYSIS OF IONIC CHANGES DURING TURION FORMATION

In view of the fact that ABA has been implicated in rapid events such as stomatal closure which in turn is thought to be regulated by rapid changes in ion fluxes, could the changes in protein synthesis result from a selective effect of ABA on the permeability of the plasmalemma (perhaps by way of a membrane bound receptor) as has been demonstrated in the development of certain animal systems upon fertilisation (Jaffe, 1969; Steinhardt et al., 1977)? If the initial action of ABA was on ion fluxes, as has often been suggested, which in turn controlled other cellular processes, one would expect these changes to be not only rapid but dramatic.

This preliminary study began with the simple measurement of the inorganic composition of fronds and turions of S. polyrrhiza. This was followed by an analysis of the individual ion fluxes and compartment concentrations not only in fronds and turions, but also in fronds treated with ABA.

6.2 THE INORGANIC COMPOSITION OF S. POLYRRHIZA FRONDS AND TURIONS

The inorganic composition of control plantlets, and turions and associated mother fronds produced by 10 days incubation with 1×10^{-7}M ABA is shown in table 12. When the results were expressed on a fresh weight basis there is a large increase in all the minerals studied in the turion of S. polyrrhiza (N) (Table 12a). This increase is especially noticeable when the molarity of the elements is calculated (Table 12b) on the basis that only approximately 44% of the turion is water. On a dry weight basis of course, the concentration of the inorganic constituents was either decreased or

Table 12: The inorganic composition of fronds, and mother fronds and turions of S. polyrrhiza (N) incubated in 1×10^{-7} M ABA

(a) Inorganic composition (μmole. g fresh weight^{-1})

Sample	ABA (M)	Phosphorus	Iron	Calcium	Sodium	Potassium	Magnesium
Control fronds	-	53	0.31	7.9	20	55	20
Mother fronds	1×10^{-7}	39	0.49	15.3	22	53	25
Turions	1×10^{-7}	348	2.42	20.6	117	162	182

(b) Inorganic composition (mM)

Sample	ABA (M)	Phosphorus	Iron	Calcium	Sodium	Potassium	Magnesium
Control fronds	-	58	0.34	8.6	22	60	22
Mother fronds	1×10^{-7}	42	0.53	16.6	24	58	27
Turions	1×10^{-7}	777	5.40	46.0	261	362	406

(c) Inorganic composition (μmole. g dry weight^{-1})

Sample	ABA (M)	Phosphorus	Iron	Calcium	Sodium	Potassium	Magnesium
Control fronds	-	679	4.00	101	256	705	256
Mother fronds	1×10^{-7}	366	4.60	144	207	498	235
Turions	1×10^{-7}	489	3.40	29	165	288	256

remained unchanged in the turion compared to control plantlets (Table 12c).

Although ABA decreased the P/Fe and K/Ca ratios significantly in the mother fronds, as has been found for L. gibba (DeKock et al., 1978), these changes were not found in the turions where both ratios were similar to those found in untreated fronds. However, it is interesting that the K/Na ratio decreased from 4·63 in control fronds to 4·06 in ABA treated mother fronds down to 2·34 in the turions. It has been suggested that a high K/Na ratio in the cytoplasm is essential for the activity of many enzymes, in particular for those involved in protein synthesis (Lubin, 1964). ABA was also found to decrease the K/Na ratio and selectivity of uptake in favour of Na in beetroot discs (van Steveninck, 1972). The differences found between the inorganic constituents of control fronds and turions led to the study of individual ion fluxes and compartment contents by the method of compartmental analysis.

6.3 COMPARTMENTAL ANALYSIS OF ION FLUXES AND COMPARTMENT CONTENTS

The measurement of the kinetics of isotope exchange between a radioactively loaded tissue and a non-labelled external solution offers an indirect way for compartmental analysis i.e. for the analysis of concentrations in particular compartments and of fluxes at the boundaries of such compartments (i.e. membranes). The basis of this method is described below.

Plantlets or turions were immersed in a radioactively labelled ion solution for eighteen hours, where the tissue takes up ions from the solution until it is presumably in a steady state. In this

situation influx and efflux should be equal and constant with time, so that the ion content for the duration of the experiment should not change. After radioactive loading, the tissue was transferred into a non-labelled ion solution having the same concentration and composition as the solution used for labelling, and the kinetics of the efflux of the isotope was measured over a period of 9 hours. From counts of activity remaining in the tissue at the end of elution and from the activity in each of the washings, an efflux curve was constructed for each ion (e.g. Fig. 68) and analysed in the manner of Macklon and Higinbotham (1970).

The curve obtained can be considered as a compound curve, made up of first order rate losses from each compartment in the tissue. This yields a series of straight lines (from semi-logarithmic plots) each characteristic of a particular phase. The contribution of the slowest compartment, assumed here to be the vacuole, is indicated by the final linear component of the curve (Fig. 68). The line resulting has a slope which gives the rate constant k_v for the loss from the vacuole and the intercept at zero time gives the amount of radioactivity in the vacuole at the start of efflux (I_v).

Subtraction of the vacuolar component from the activity of the total tissue at each time interval yields an efflux curve representing efflux from all compartments except the vacuole (Fig. 69), with the final linear phase being attributed to the cytoplasm. Further analysis in this way, reveals 3 additional phases for Ca^{2+} (Figs. 70 and 71) and 2 phases for Na^+, Cl^- and K^+ (Fig. 75). The 2 remaining phases for the monovalent ions are considered to relate to the apoplastic free space (AFS) in the tissue and the superficial film

Figure 68

Time course of reduction in the amount of ^{45}Ca per gram of control fronds of S. polyrrhiza (N) during elution in unlabelled H/2 medium. Final linear phase attributed to the vacuole.

FIGURE 68

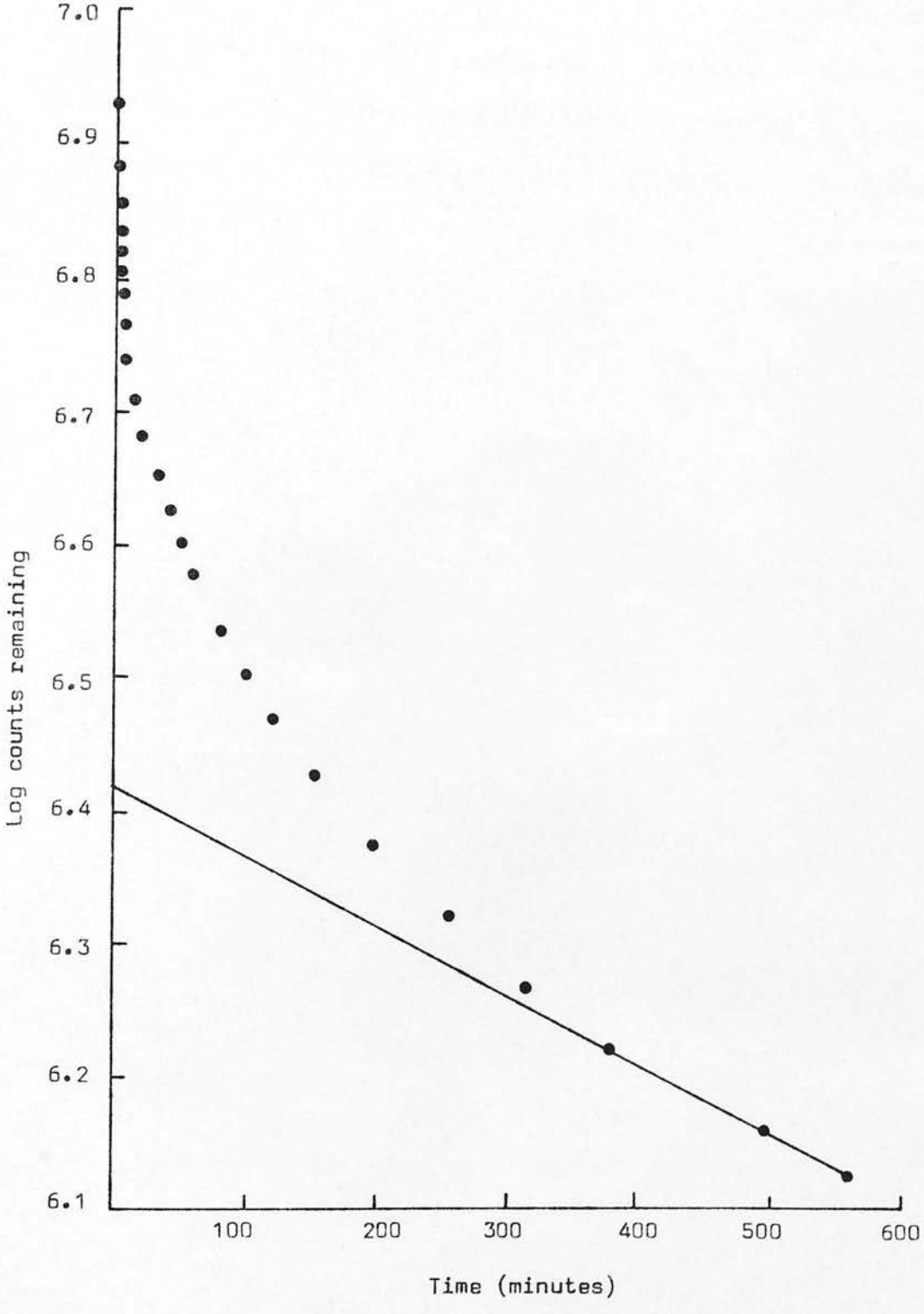

7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
Log counts remaining
100
200
300
400
500
600
Time (minutes)

Figure 69

Time course of reduction in the amount of ^{45}Ca per gram of control fronds of S. polyrrhiza during elution in unlabelled H/2 medium. Reduction in the amount of ^{45}Ca estimated to be in the cytoplasm, free space and superficial film. Final linear phase attributed to the cytoplasm.

FIGURE 69

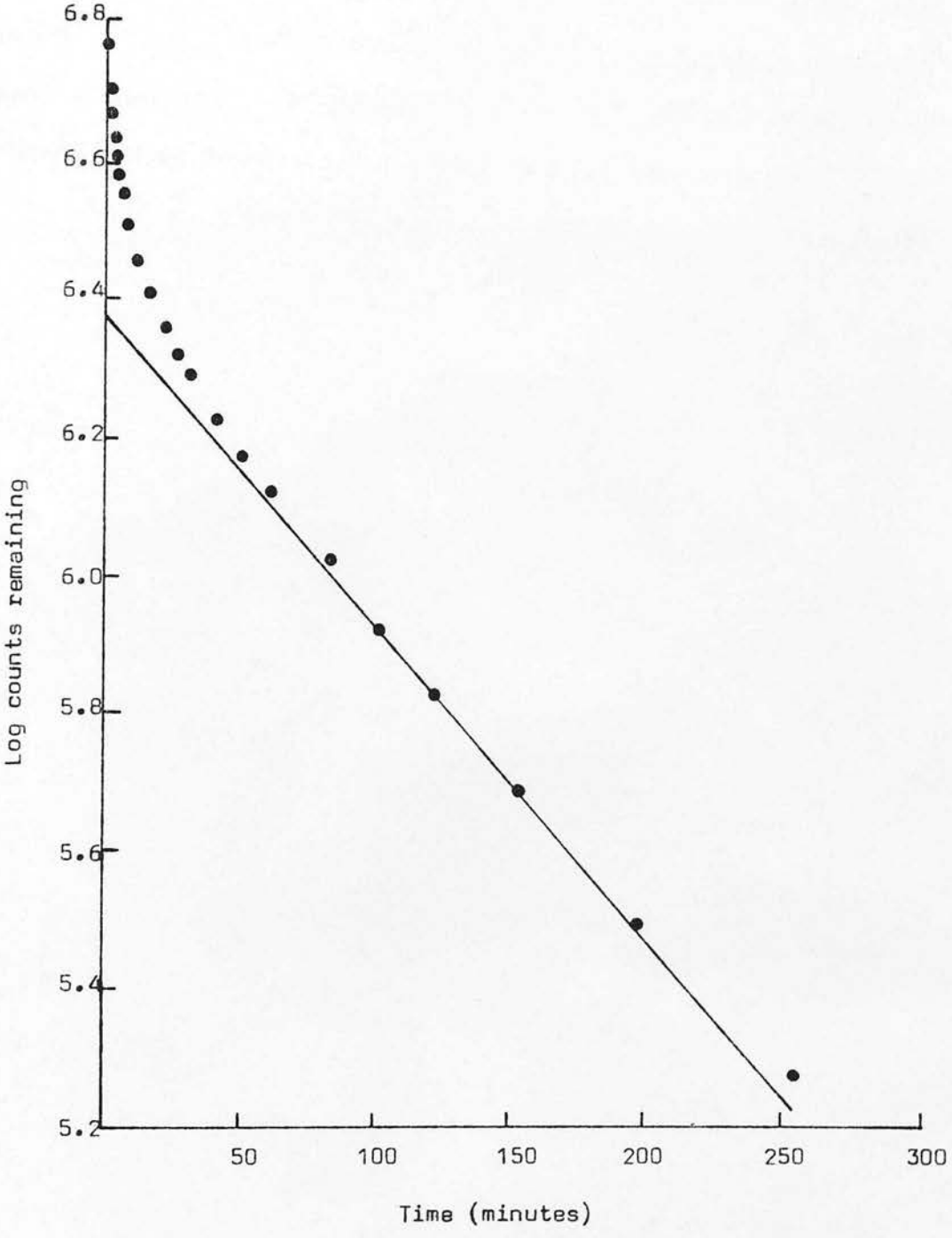

6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
Log counts remaining
50
100
150
200
250
300
Time (minutes)

Figure 70

Time course of reduction in the amount of ^{45}Ca per gram of control fronds of S. polyrrhiza (N) during elution in unlabelled H/2 medium. Reduction in the amount of ^{45}Ca estimated to be in the Donnan free space, water free space and superficial film. Final linear phase attributed to the DFS.

FIGURE 70

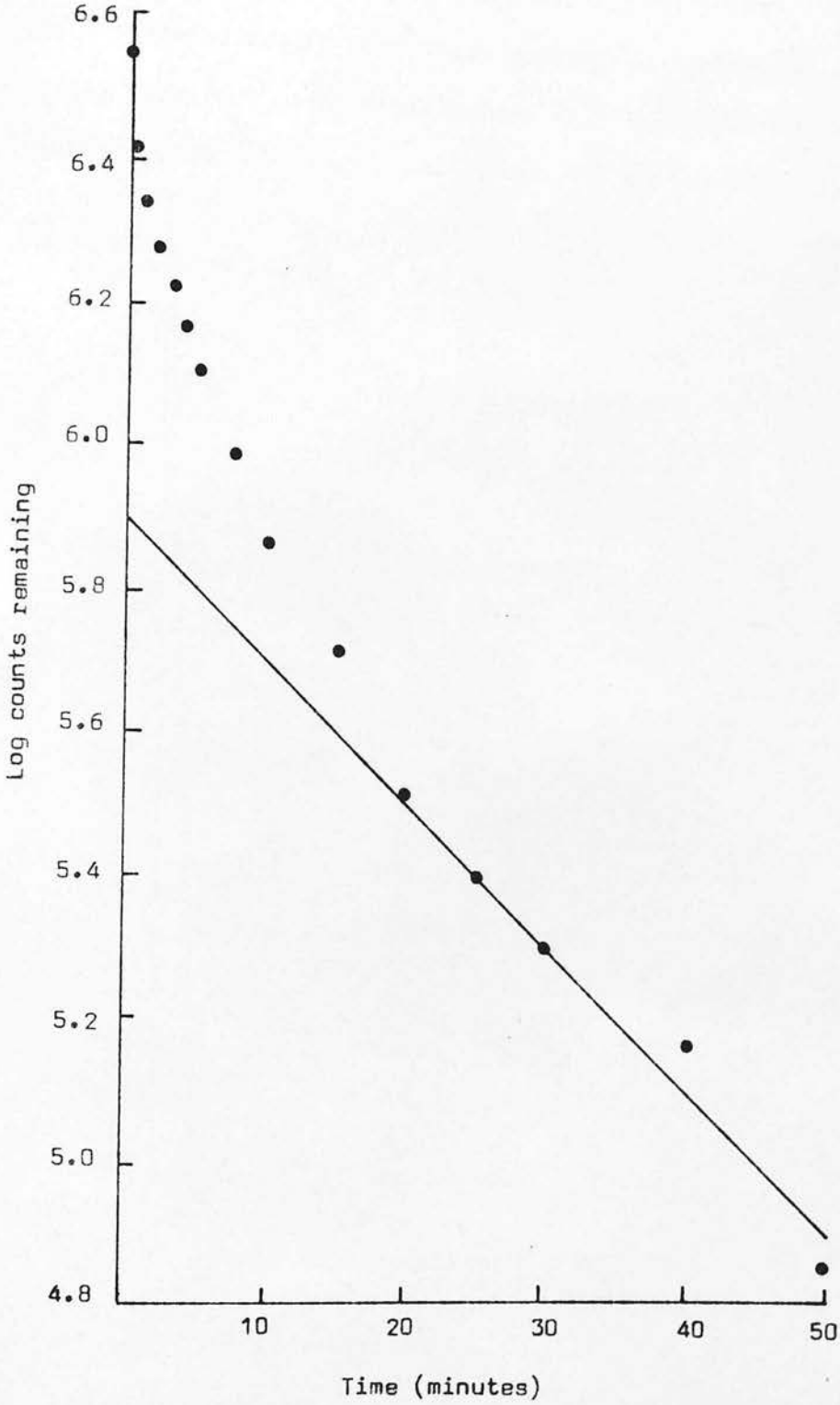

6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
Log counts remaining
10
20
30
40
50
Time (minutes)

Figure 71

Time course of reduction in the amount of ^{45}Ca per gram of control fronds of S. polyrrhiza (N) during elution in unlabelled H/2 medium. Reduction in the amount of ^{45}Ca estimated to be in the water free space and superficial film (●) and in the superficial film alone (o).

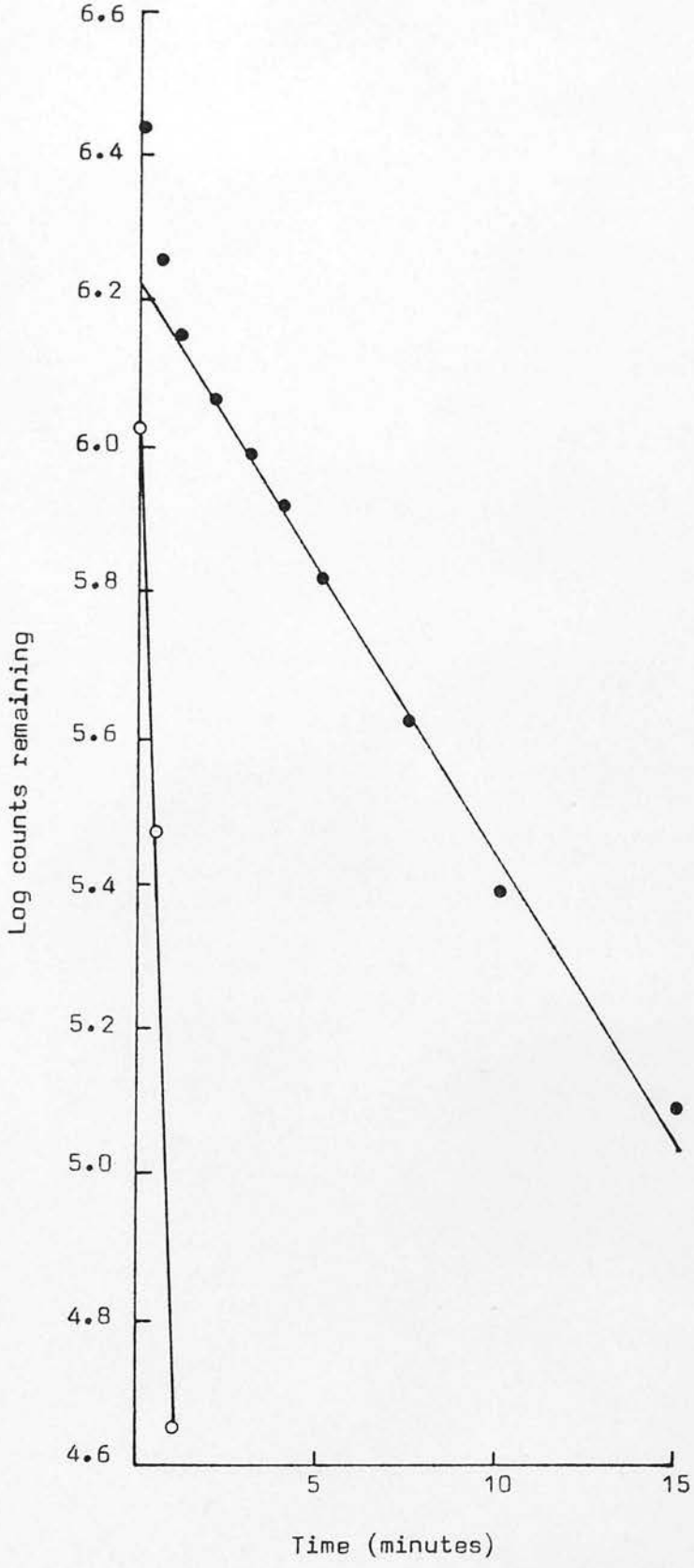

6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
Log counts remaining
5
10
15
Time (minutes)

FIGURE 71

of solution on the fronds or turions. With Ca^{2+}, the extra component has a rate constant between that associated with cytoplasm efflux ($t_{\frac{1}{2}}$ about an hour) and that related to exchange in the water free space (WFS) ($t_{\frac{1}{2}}$ less than 5 min), and it has been proposed (Macklon, 1975b) that it is associated with fixed negative charges in the cell walls which bind divalent cations preferentially to monovalent cations. This compartment is known as the Donnan free space (DFS).

From the graphical analysis of the results, the efflux constant (k) and half-time for exchange ($t_{\frac{1}{2}}$) can be estimated for each phase. In calculating the apparent amount of each ion in each compartment at the start of the washing procedure, it is assumed that the cytoplasm and free spaces reach a steady state of specific activity well before the end of the 18 hour loading period and that the 'apparent' amount of the ion in question in each compartment can be taken as equal to the counts per minute at the intercept, divided by the specific activity of the external solution. The amount of a particular ion in the vacuole (Q_v), is then represented by the difference between the total estimated chemically and the sum of the ion in the other compartments by specific activity. Finally, apparent influx (I_v/t_{up}) across the tonoplast is estimated from the amount of isotope accumulated in the vacuole (I_v) during the loading period (t_{up}) during which apparent influx and net flux should be approximately linear, as well as during the elution period.

Net uptake is one factor which introduces uncertainty into flux analysis of this type, since it leads to an increase in the content of the vacuole during the experiment. Nevertheless, this method has

been used in studies in which net uptake of the ions under study was proceeding at a relatively high rate (Macklon and Higinbotham, 1970; Pierce and Higinbotham, 1970). For this reason the apparent and net uptake of each ion are shown in figures 72, 76, 77 and 78, and were borne in mind when interpreting the results.

The efflux and content values obtained from such graphical analysis are apparent values i.e. values are not corrected for concurrent opposing fluxes. They considerably underestimate cytoplasmic content in particular, since in basing them on data for the appearance of tracer in the washing medium, no account of the concomitant transfer of ions between the cytoplasm and the vacuole is taken. Nevertheless, all the data are present to estimate real values for the fluxes and for the ion content of the cytoplasm and the vacuole, using the following equations, where J is the ion flux in the direction indicated by the subscripts o (outside), c (cytoplasm) and v (vacuole).

$$J_{oc} = k_c I_c + \frac{I_v}{t_{up}} \quad \cdots\cdots(1)$$

where k_c is the efflux constant for the second slowest phase believed to be the cytoplasm, I_c is the apparent content of this phase, and I_v/t_{up} is the apparent influx to the vacuole.

$$J_{vc} = J_{oc} \cdot \frac{k_v Q_v}{k_c I_c} \quad \cdots\cdots(2)$$

Q_v, the amount of ion in the vacuole, is the amount determined for the total tissue less the apparent contents of the other compartments.

$$J_{co} = k_c I_c + k_v Q_v \quad \cdots\cdots(3)$$

where k_v is the efflux constant of the slowest phase, the vacuole.

Here it is assumed that the ion content is steady, so that

$$J_{cv} = J_{oc} + J_{vc} - J_{co} \quad \ldots\ldots\ldots\

elution, this proved to be too short a time to be confident that full equilibrium was established by the time elution started. Strictly speaking then, both content and fluxes may have been changing during loading and elution. This objection would be least important for Ca^{2+} since the concentration of this ion in the 2 media was quite similar. The results can be seen to be very similar to those obtained in Hutner's medium, and the time course of net uptake shows a fairly constant value (i.e. no appreciable uptake) and one is justified in treating Ca^{2+} flux data on the basis that $J_{in} = J_{out}$.

Another problem that must be recognised is that control fronds may be growing in Hutner's medium (less so in 1X), whereas turions will not (as long as ABA is present), and the very different nature of the turion and the control fronds (air space formation, differentiation of the mesophyll etc.) makes a direct comparison between the 2 types of tissue difficult. However, in some of the experiments the effect of short and long exposures of fronds to ABA have also been studied. These experiments are not subject to the caution outlined above.

6.3.1 Ca^{2+} fluxes and contents in control fronds and turions in H/2 medium.

The efflux curves from the Ca^{2+} efflux experiment are shown in figures 68, 69, 70 and 71 for control fronds. No further figures are presented for the sake of brevity. The primary data are presented in table 13.

For each of the 5 phases, values are given for efflux constants, half-times of exchange and apparent contents. When the apparent

Table 13: Efflux constants (k), half-times for exchange ($t_{\frac{1}{2}}$) and apparent contents for Ca^{2+} in each compartment, and apparent influx to vacuole (I_v/t_{up}). Control fronds of S. polyrrhiza (N) eluted in H/2 medium, and turions eluted in H/2 + 1 x 10^{-7}M ABA.

Compartment	Tissue	k (s^{-1})	$t_{\frac{1}{2}}$ (min)	Apparent content (μmole. g^{-1})	I_v / t_{up} (μmole. g^{-1}. h^{-1})
Vacuole	fronds	1.992×10^{-5}	579.8	4.448	0.078
	turions	3.154×10^{-5}	366.2	8.364	0.120
Cytoplasm	fronds	1.758×10^{-4}	65.7	1.982	-
	turions	2.262×10^{-4}	51.1	6.588	-
Donnan free space	fronds	7.592×10^{-4}	15.2	0.394	-
	turions	9.081×10^{-4}	12.7	2.999	-
Water free space	fronds	2.948×10^{-3}	3.9	0.845	-
	turions	3.144×10^{-3}	3.7	1.109	-
Superficial	fronds	4.694×10^{-2}	0.25	0.538	-
	turions	3.128×10^{-2}	0.37	0.804	-

contents are expressed as a % of the total apparent content, they compare very favourably with estimates of the compartment volumes by stereological analysis (section 3.7). The Donnan free space compartment in particular is much higher in the turion than in the control fronds, possibly corresponding to the increase in the volume of the cell wall found in the turion by stereological analysis.

Real fluxes and contents of Ca^{2+}, calculated from the apparent values of table 13 using the equations (1) to (5), are shown in table 14a. The values were calculated on the assumption that all of the calcium not identified as exchangeable in the cytoplasm and free space (4·4 μmole. g^{-1} for fronds and 8·4 μmole. g^{-1} for turions) was in an ionised form in the vacuole (max value). There is no direct evidence that this is correct however, and so the results were also calculated using a minimum value of vacuolar Ca^{2+} content. The minimum content of the vacuole was taken as the product of apparent influx and loading time i.e. I_v. This estimate, amounting to 1·32 μmole. g^{-1} for control fronds and 2·04 μmole. g^{-1} for turions, would imply that a large proportion of calcium in the tissue was in a non-exchangeable and non-ionised form either in the vacuole (e.g. calcium oxalate crystals) or in the cytoplasm and the wall.

It is difficult to establish the proportion of total calcium in the tissue which contributes to the chemical activity in the vacuole. This can be measured directly in the giant algae (Spanswick and Williams, 1965) and was found to be completely ionised. Ca^{2+} in the vacuole of Spirodela tissue can only be estimated within rather wide limits. In onion root segments, Macklon (1975b) showed that a consideration of the flux ratios calculated on the basis of each of

Table 14: Unidirectional fluxes and contents of Ca^{2+} computed from the results in Table 13, for control fronds and turions of S. polyrrhiza (N)

	Compartment	Tissue	Influx (μmole. g^{-1}. h^{-1})	Efflux (μmole. g^{-1}. h^{-1})	Flux ratio	Content (μmole. g^{-1})
(a)	Vacuole	fronds (max)	0.110	0.351	0.31	4.448
		fronds (min)	0.087	0.104	0.84	1.322
		turions (max)	0.149	0.978	0.15	8.364
		turions (min)	0.128	0.239	0.54	2.042
	Cytoplasm	fronds (max)	0.842	1.084	0.78	1.982
		fronds (min)	0.842	0.859	0.98	1.496
		turions (max)	4.203	5.031	0.84	6.588
		turions (min)	4.203	4.314	0.97	5.454
(b)	Vacuole	fronds	0.086	0.086	1.00	1.084
		turions	0.124	0.124	1.00	1.058
	Cytoplasm	fronds	0.842	0.842	1.00	1.466
		turions	4.203	4.203	1.00	5.313

these estimates could lead to the true value for dissociated Ca^{2+} in the vacuole. This value lay at an intermediate point between the maximum and minimum values. If net flux is zero, the flux ratio must be 1 and

$$k_v Q_v = I_v / t_{up} \quad \cdots\cdots\cdots\

table 15a with the fluxes expressed per unit area of membrane.

It is quite clear that there is far more Ca^{2+} in the cytoplasm of the turion cells than in control frond cells, and fluxes of Ca^{2+} at the plasmalemma are far higher in the turion than in the control fronds. Could the higher content of Ca^{2+} in the cytoplasm of the turion have arisen by an initial action of ABA on the developing turion? To see if simple addition of ABA to fronds of S. polyrrhiza had any effect on the fluxes or contents of Ca^{2+}, the above experiment was repeated on control fronds and fronds treated for 18 hours with 1×10^{-7} M ABA.

6.3.2 The effect of ABA on Ca^{2+} fluxes and contents in plantlets of S. polyrrhiza in H/2.

Table 16 shows the efflux constants, half-times for exchange and apparent contents for Ca^{2+} in each compartment, derived from the efflux curves.

The primary data indicated that ABA had very little effect on either Ca^{2+} fluxes or contents. Real fluxes and contents of Ca^{2+}, calculated from the apparent values of table 16 are shown in table 17. Using the cell compartment volumes and membrane areas found by stereological analysis, the contents and fluxes given in table 17b were used to compute the cytoplasm and vacuole Ca^{2+} concentrations and the fluxes at both membranes (Table 15b).

There appears to be little effect of ABA on the Ca^{2+} fluxes or contents of Ca^{2+} on plantlets of S. polyrrhiza in H/2 medium. However, since whole plantlets were used, any differential effect of ABA on the Ca^{2+} fluxes in the young developing turions might be

Table 15: Chemical concentrations of Ca^{2+} in the cytoplasm and the vacuole of control fronds and turions of S. polyrrhiza (N) and fluxes across the plasmalemma and the tonoplast.

Table 15b shows the effect of ABA on the control fronds.

	Tissue	Concentration in cytoplasm (mM)	Plasmalemma Flux (μmole. cm^{-2}. s^{-1})	Concentration in vacuole (mM)	Tonoplast Flux (μmole. cm^{-2}. s^{-1})
(a)	Control fronds	7•97	1•193	2•22	0•138
	Turions	26•62	5•274	2•84	0•164
(b)	Control fronds	8•22	1•074	2•88	0•218
	Fronds + ABA	7•70	1•070	2•46	0•196

Table 16: Efflux constants (k), half-times for exchange ($t_{\frac{1}{2}}$) and apparent contents for Ca^{2+} in each compartment, and apparent influx to the vacuole (I_v/t_{up}). Control fronds and fronds incubated

Compartment	Tissue	k (s^{-1})	$t_{\frac{1}{2}}$ (min)	Apparent content (μmole. g^{-1})	I_v / t_{up} (μmole. g^{-1}. h^{-1})
Vacuole	control	2.282×10^{-5}	506	8.290	0.115
	ABA	2.420×10^{-5}	477	8.231	0.105
Cytoplasm	control	1.644×10^{-4}	70	1.086	-
	ABA	1.719×10^{-4}	67	1.050	-
Donnan free space	control	9.404×10^{-4}	12.3	0.516	-
	ABA	1.102×10^{-3}	10.5	0.529	-
Water free space	control	8.982×10^{-3}	1.3	0.695	-
	ABA	1.075×10^{-2}	1.1	0.702	-
Superficial	control	4.165×10^{-2}	0.28	1.913	-
	ABA	3.577×10^{-2}	0.32	1.988	-

in 1×10^{-7} M ABA of S. polyrrhiza (N) eluted in H/2 medium.

Table 17: Unidirectional fluxes and contents of Ca^{2+} computed from the results in Table 16, for control fronds and fronds incubated in 1×10^{-7} M ABA of S. polyrrhiza (N).

	Compartment	Tissue	Influx (μmole. g^{-1}. h^{-1})	Efflux (μmole. g^{-1}. h^{-1})	Flux ratio	Content (μmole. g^{-1})
(a)	Vacuole	control (max)	0·229	0·745	0·31	7·692
		control (min)	0·146	0·201	0·73	2·078
		ABA (max)	0·213	0·780	0·27	7·706
		ABA (min)	0·131	0·191	0·69	1·885
	Cytoplasm	control (max)	0·758	1·275	0·60	1·684
		control (min)	0·758	0·814	0·93	1·529
		ABA (max)	0·755	1·321	0·57	1·575
		ABA (min)	0·755	0·814	0·93	1·431
(b)	Vacuole	control	0·136	0·136	1·00	1·405
		ABA	0·122	0·122	1·00	1·202
	Cytoplasm	control	0·758	0·758	1·00	1·512
		ABA	0·755	0·755	1·00	1·416

masked by the large quantities of mother tissue present. Moreover, compartmental analysis would not be a suitable method for the study of isolated developing turions during their development as has been used to study other developmental systems (Mallery, 1979), since dissection of the young primordia would invariably lead to leakage of ions from the damaged tissue which would be exposed via the stolon.

Although the half-times for exchange of Ca^{2+} in the vacuole of control fronds is lower, and in the cytoplasm higher than those obtained for onion root segments in 1X medium (Macklon, 1975b), generally the 2 types of tissue are rather similar in Ca^{2+} content and flux rates across the membranes. However, in onion root segments the actual content of the vacuole lay between the theoretical upper and lower limits of Q_v and therefore more calcium was in an ionised form. This difference might be explained by the abundance of calcium oxalate crystals in the vacuoles of both fronds and turions of S. polyrrhiza.

The 2 experiments outlined above were combined in one experiment to compare the Ca^{2+} fluxes in control fronds, fronds treated for 1 hour with ABA, fronds treated with ABA for 18 hours and turions. However, the entire experiment was performed with 1X solution.

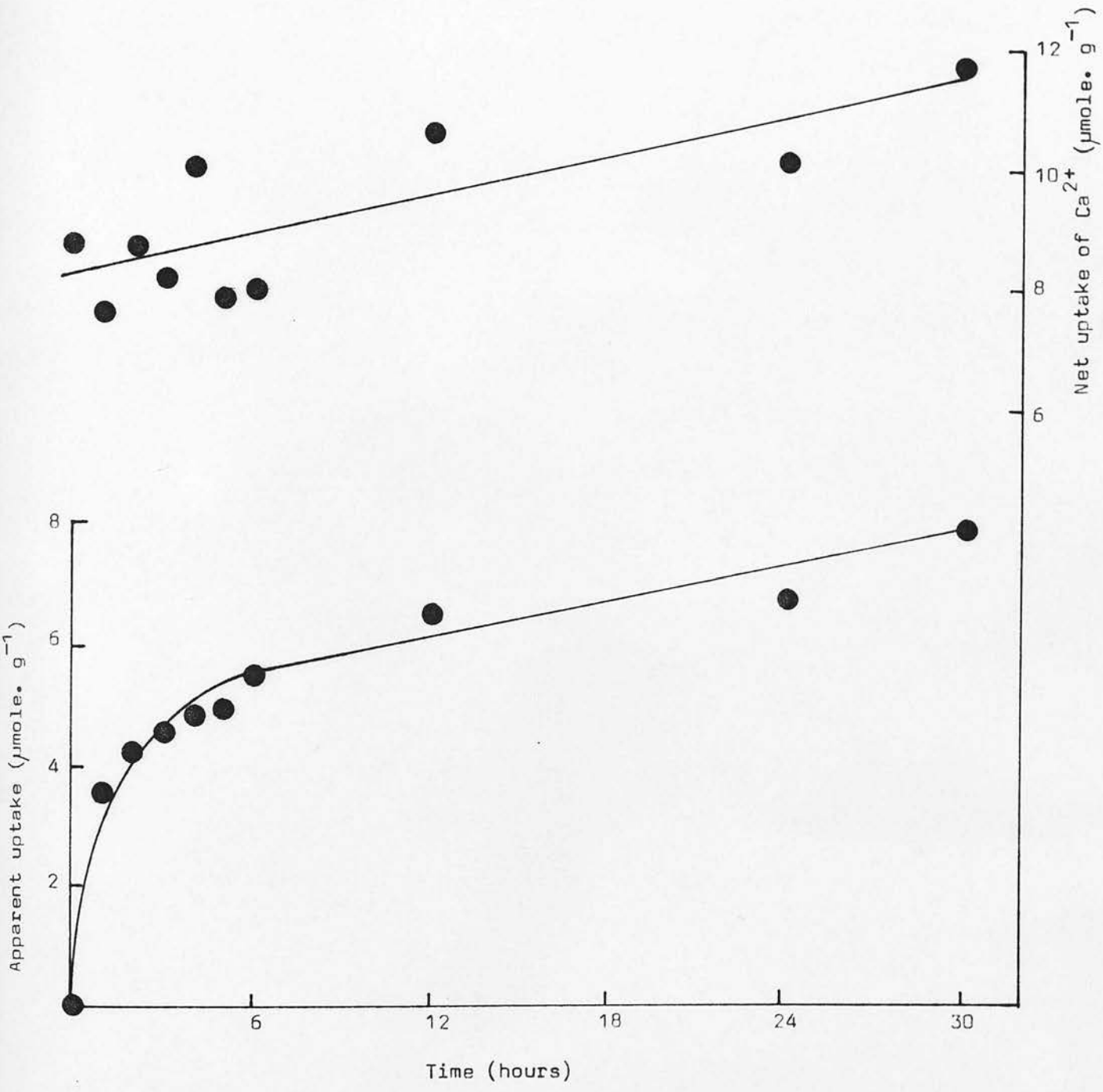
6.3.3 Ca^{2+} fluxes and contents in fronds and turions, and the effect of long and short exposures of fronds to ABA.

In this work Ca^{2+} uptake from a radioactively labelled solution of 1X was performed in parallel with the elution, in order to establish the linear nature of apparent and net uptake (Fig. 72).

Figure 72

Apparent uptake of Ca^{2+} as a function of time (left hand ordinate) and Net uptake of Ca^{2+} as a function of time in control fronds of *S. polyrrhiza* (N) in 1X solution.

FIGURE 72


Net uptake of Ca^{2+} (μmole. g^{-1})
12
10
8
6
Apparent uptake (μmole. g^{-1})
8
6
4
2
6
12
18
24
30
Time (hours)

The steep part of the apparent uptake curve is related to exchange in the outer compartments and is a characteristic feature of Ca^{2+} uptake (Dunlop, 1973). Exchange occurs between the uptake solution and the surface film on the fronds, the WFS, the DFS and the cytoplasm, and approaches completion in each compartment after a period exceeding 5 half-times of exchange for that compartment (Pitman, 1963). Hence all these compartments are fully equilibrated with the outside solution after 5 - 6 hours, and the linear part of the apparent uptake curve represents exchange across the tonoplast (I_v/t_{up} = 0·111 μmole. g^{-1}. h^{-1}). Some net uptake did occur in the fronds, but this was only slight and was linear. It is unlikely that there was any net uptake in the turions, since they would not have been growing during the experiment.

Each experimental sample i.e. control fronds, 1 hour ABA, 18 hours ABA and turions was studied in duplicate by means of separate experiments. Table 18 shows the primary data obtained, and real fluxes and contents are shown in Table 19. It can be seen once again that there is no effect of ABA on the fluxes or contents of Ca^{2+} in plantlets of S. polyrrhiza, either after 1 or 18 hours. This is in agreement with the results obtained in Hutner's medium, and as with H/2, the Ca^{2+} fluxes of the turion are radically different from those of the vegetative fronds. When the fluxes and contents are corrected for membrane areas and compartment volumes (Table 20), it can be seen that the turions show reduced exchange across the tonoplast and a low vacuolar content relative to the vegetative fronds, and a much increased exchange across the plasmalemma with a corresponding high Ca^{2+} concentration in the

Table 18: Efflux constants (k), half-times for exchange ($t_{\frac{1}{2}}$) and apparent contents of Ca^{2+} in each compartment, and apparent influx to vacuole (I_v/t_{up}). Control fronds, eluted with 1X solution, fronds + 1 hr ABA, fronds + 18 hr ABA, and turions eluted with 1X solution and 1×10^{-7}M ABA.

Compartment	Tissue	k (s^{-1})	$t_{\frac{1}{2}}$ (min)	Apparent content (μmole. g^{-1})	I_v / t_{up} (μmole. g^{-1}. h^{-1})
Vacuole	control	1.907×10^{-5}	608	4.933	0.097
	1 hr ABA	1.752×10^{-5}	694	3.974	0.099
	18 hr ABA	2.417×10^{-5}	478	4.463	0.111
	turions	4.584×10^{-5}	265	2.912	0.033
Cytoplasm	control	1.928×10^{-4}	60	2.687	-
	1 hr ABA	1.575×10^{-4}	60	2.794	-
	18 hr ABA	1.998×10^{-4}	58	3.040	-
	turions	2.707×10^{-4}	43	7.901	-
Donnan free space	control	7.946×10^{-4}	14.6	0.600	-
	1 hr ABA	1.011×10^{-3}	11.5	0.621	-
	18 hr ABA	7.895×10^{-4}	16.3	0.534	-
	turions	7.200×10^{-4}	17.0	5.149	-
Water free space	control	6.839×10^{-3}	1.8	1.075	-
	1 hr ABA	7.580×10^{-3}	1.6	1.068	-
	18 hr ABA	6.429×10^{-3}	1.9	1.022	-
	turions	4.486×10^{-3}	2.6	2.721	-
Superficial	control	4.734×10^{-2}	0.25	3.033	-
	1 hr ABA	5.090×10^{-2}	0.23	3.505	-
	18 hr ABA	4.922×10^{-2}	0.24	3.249	-
	turions	3.705×10^{-2}	0.32	2.929	

Table 19: Unidirectional fluxes and contents of Ca^{2+} computed from the results in Table 18, for control fronds, fronds + 1 hour ABA, fronds + 18 hour ABA and turions of S. polyrrhiza (N) eluted in

(a)

Compartment	Tissue	Influx (μmole. g^{-1}. h^{-1})	Efflux (μmole. g^{-1}. h^{-1})	Flux ratio	Content (μmole. g^{-1})
Vacuole	control (max)	0.114	0.333	0.34	4.628
	control (min)	0.104	0.128	0.81	1.791
	1 hr ABA (max)	0.116	0.261	0.44	3.813
	1 hr ABA (min)	0.107	0.121	0.88	1.850
	18 hr ABA (max)	0.130	0.391	0.33	4.276
	18 hr ABA (min)	0.121	0.191	0.63	2.087
	turions (max)	0.052	0.588	0.09	2.912
	turions (min)	0.048	0.167	0.29	0.827
Cytoplasm	control (max)	1.961	2.181	0.90	2.992
	control (min)	1.961	1.985	0.99	2.976
	1 hr ABA (max)	1.687	1.832	0.92	3.197
	1 hr ABA (min)	1.687	1.701	0.99	3.173
	18 hr ABA (max)	2.298	2.559	0.90	3.377
	18 hr ABA (min)	2.298	2.368	0.97	3.362
	turions (max)	7.425	7.965	0.93	8.020
	turions (min)	7.425	7.546	0.98	8.017

1X solution.

Table 19 continued:

(b)

Compartment	Tissue	Influx (μmole. g^{-1}. h^{-1})	S.E.	Efflux (μmole. g^{-1}. h^{-1})	Flux ratio	Content (μmole. g^{-1})	S.E.
Vacuole	control	0.103	$\pm$ 0.014	0.103	1.00	1.605	$\pm$ 0.101
	1 hr ABA	0.106	$\pm$ 0.009	0.106	1.00	1.687	$\pm$ 0.517
	18 hr ABA	0.117	$\pm$ 0.010	0.117	1.00	1.272	$\pm$ 0.101
	turions	0.033	$\pm$ 0.012	0.033	1.00	0.195	$\pm$ 0.030
Cytoplasm	control	1.961	$\pm$ 0.068	1.961	1.00	2.975	$\pm$ 0.136
	1 hr ABA	1.687	$\pm$ 0.404	1.687	1.00	3.170	$\pm$ 0.039
	18 hr ABA	2.298	$\pm$ 0.024	2.298	1.00	3.356	$\pm$ 0.039
	turions	7.730	$\pm$ 0.305	7.730	1.00	7.971	$\pm$ 0.043

S.E. = standard error of the mean of 2 determinations.

Table 20: Chemical concentrations of Ca^{2+} in the cytoplasm and the vacuole of S. polyrrhiza (N) in 1X solution and fluxes across the plasmalemma and tonoplast.

Tissue	Concentration in cytoplasm (mM)	Plasmalemma Flux (μmole. cm^{-2}. s^{-1})	Concentration in vacuole (mM)	Tonoplast Flux (μmole. cm^{-2}. s^{-1})
Control	16•18	2•778	3•29	0•165
1 hr ABA	17•24	2•390	3•45	0•170
18 hr ABA	18•25	3•256	2•60	0•188
Turions	39•94	9•700	0•52	0•044

cytoplasm. The effects at the plasmalemma were also found in H/2 medium.

It therefore appears that the plasmalemma of the turion is far more permeable to Ca^{2+} compared to that of the vegetative frond cell. This increase in permeability to Ca^{2+} was not induced by ABA in plantlets even after 18 hours, and it seems unlikely, in view of the rapid effect on DNA synthesis, that ABA exerts its effect directly or initially on the membrane. It is more likely that the development of the altered membrane permeability to Ca^{2+} is a secondary consequence of ABA induced turion formation, either due to the regulation of enzymes important in membrane transport or membrane integrity or by the lack of available metabolic energy during the latter stages of turion formation.

6.3.4 K^+, Na^+ and Cl^- fluxes and contents in fronds and turions, and the effect of long and short exposures of fronds to ABA in 1X solution.

Efflux curves were contructed for each ion from counts of activity remaining in the tissue at the end of elution and in each of the washings (only one example of the curves is shown for these monovalent ions; Figs. 73, 74 and 75).

The Na^+, Cl^- and K^+ efflux curves for control tissue show a final linear phase approached within 3 hours (Na^+), 4 hours (Cl^-) and 5 hours (K^+) from the start of elution. At the same time, the net and apparent uptake of each ion varies with the ion and tissue studied. For Cl^-, apparent uptake for both tissues was linear after the initial exchange was complete, and net uptake remained fairly constant during the experiment after initial fluctuations probably

Figure 73

Time course of reduction in the amount of ^{42}K per gram of S. polyrrhiza (N) control fronds during elution in 1X solution. Reduction in total ^{42}K content. Final linear phase attributed to the vacuole.

FIGURE 73

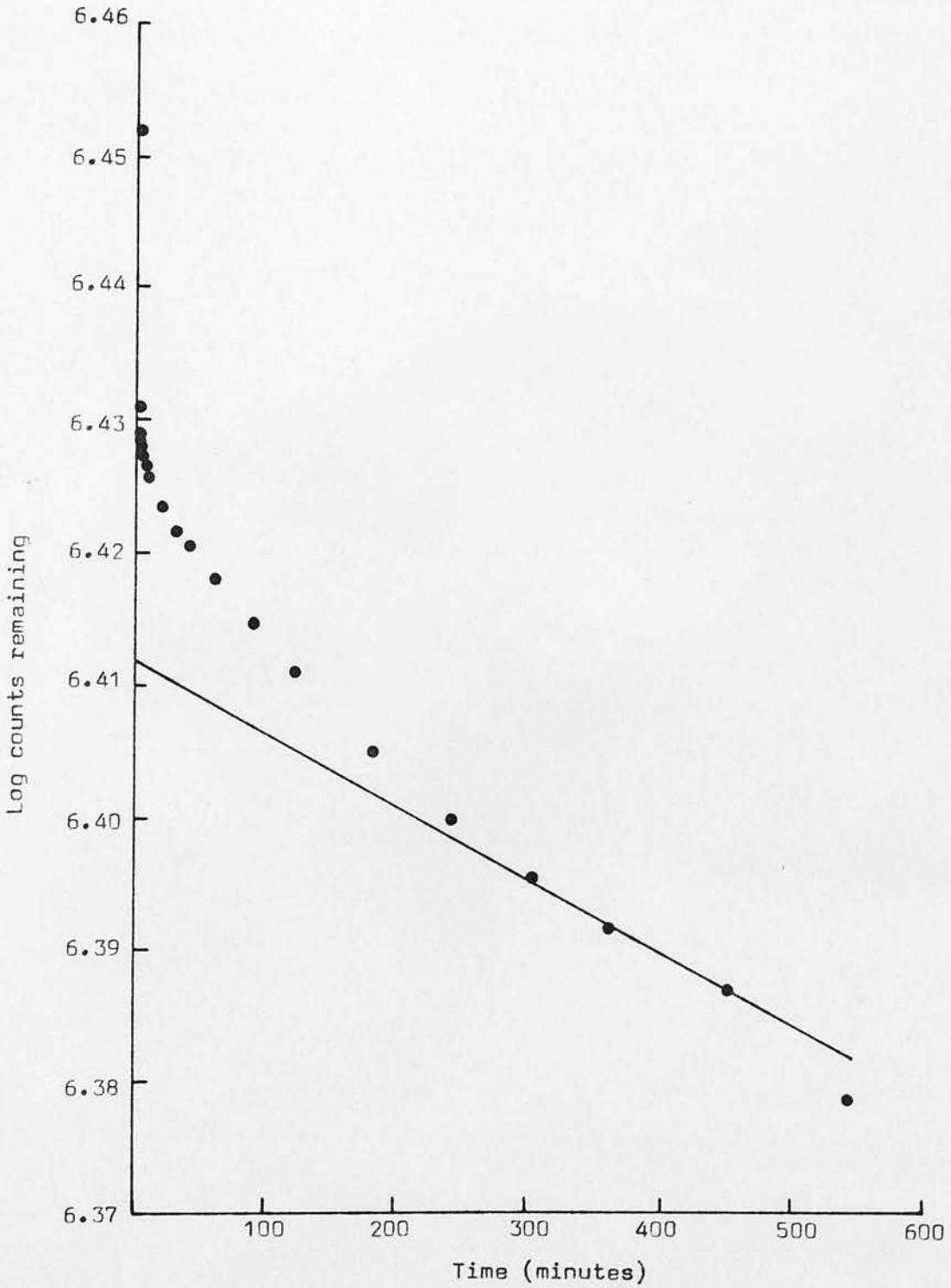

6.46
6.45
6.44
6.43
6.42
6.41
6.40
6.39
6.38
6.37
Log counts remaining
100
200
300
400
500
600
Time (minutes)

Figure 74

Time course of reduction in the amount of ^{42}K per gram of control fronds of S. polyrrhiza (N) during elution in 1X solution (unlabelled). Reduction in the amount of ^{42}K estimated to be in the cytoplasm, water free space and superficial film. Final linear phase attributed to the cytoplasm.

FIGURE 74

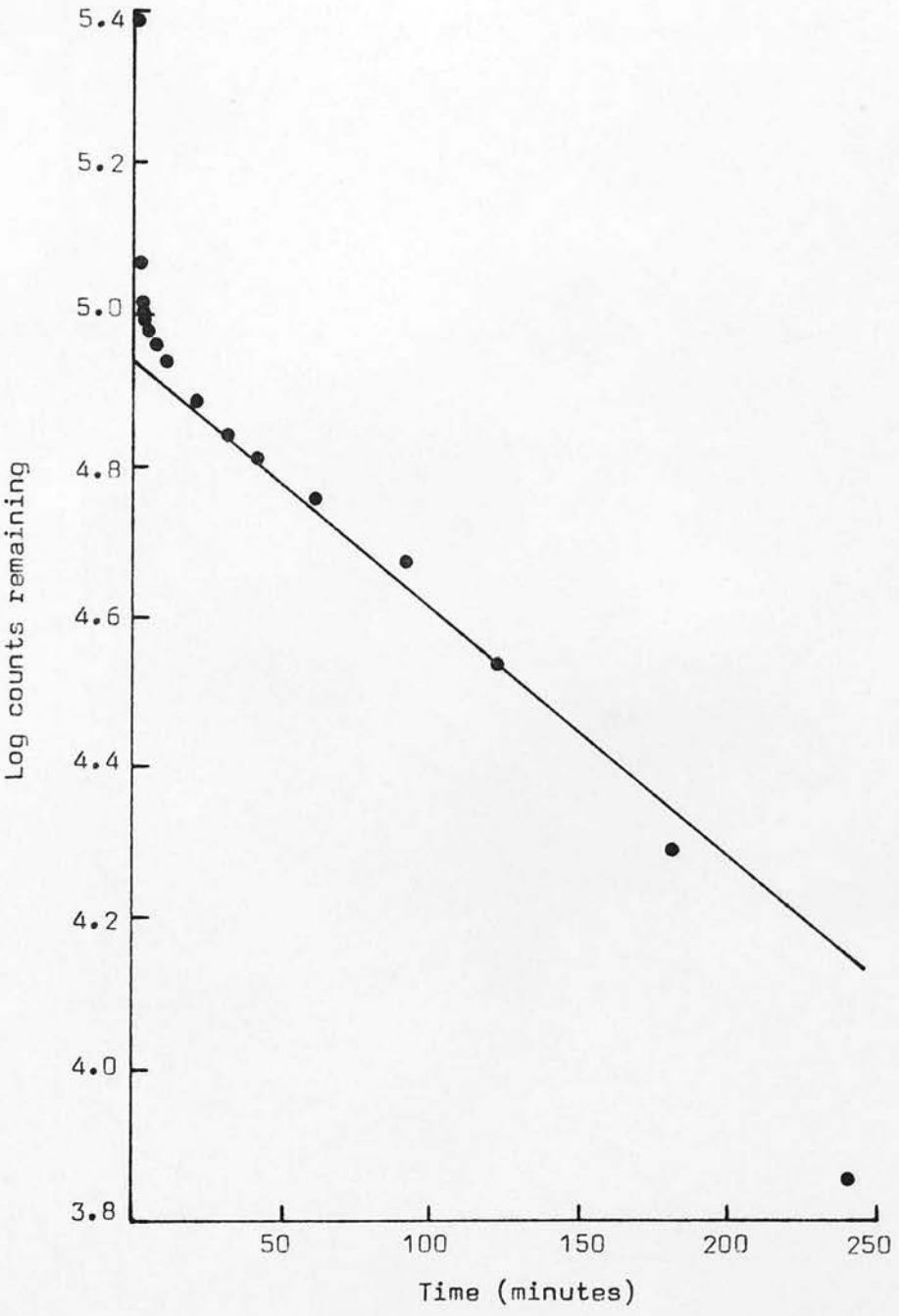
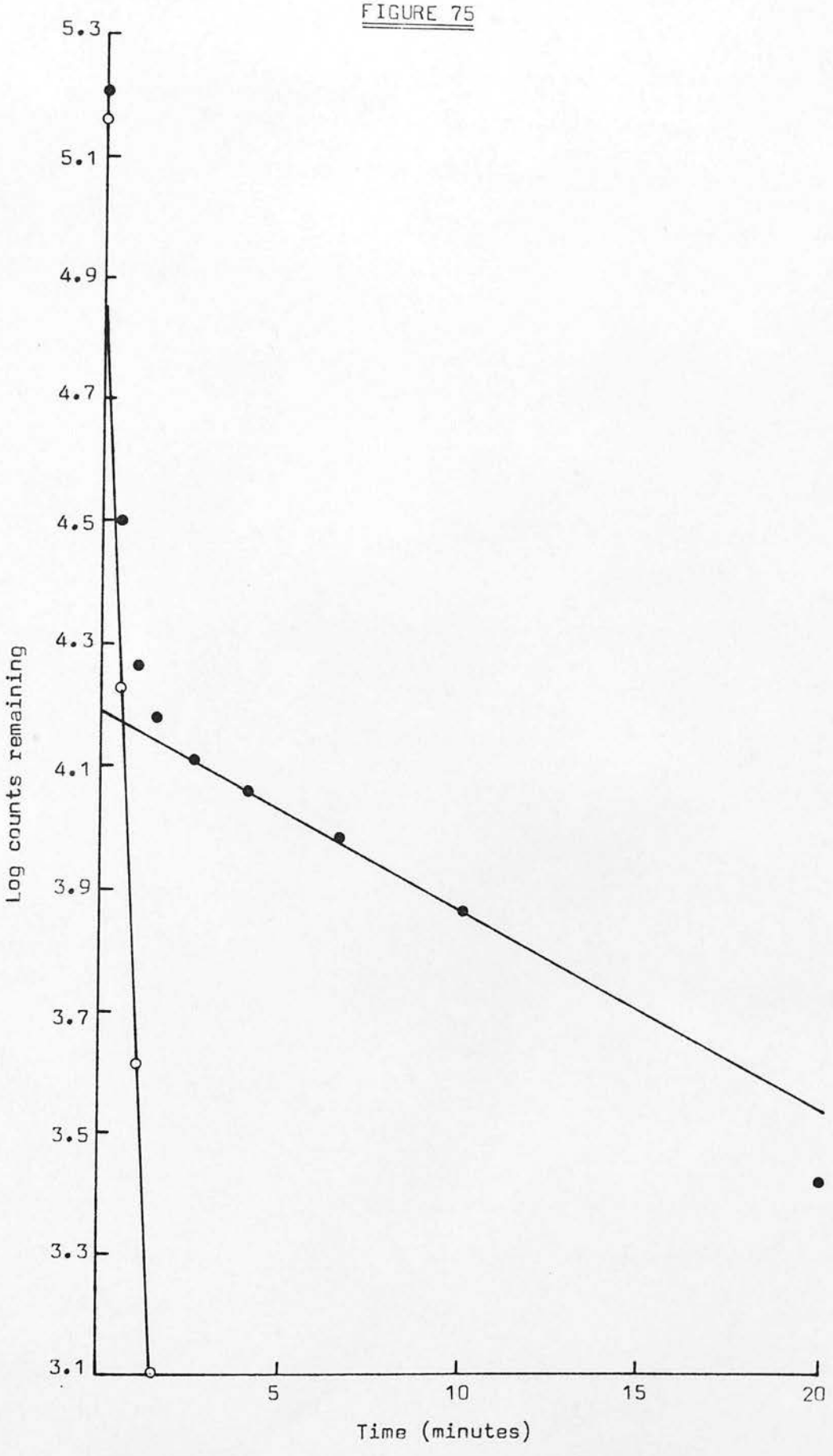

5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
Log counts remaining
50
100
150
200
250
Time (minutes)

Figure 75

Time course of reduction in the amount of ^{42}K per gram of control fronds of S. polyrrhiza (N) during elution in unlabelled 1X solution. Reduction in the amount of ^{42}K estimated to be in the water free space and superficial film (●) and in the superficial film alone (○).

FIGURE 75


5.3
5.1
4.9
4.7
4.5
4.3
4.1
3.9
3.7
3.5
3.3
3.1
Log counts remaining
5
10
15
20
Time (minutes)

due to the change in Cl^- concentration from 0•2 mM in H/2 medium to 1 mM in 1X solution (Fig. 78).

For K^+ the apparent uptake was exponential in control plantlets but linear in the turions, and net content, although fluctuating, did not alter significantly throughout the experiment (Fig. 76). Similar data were obtained for Na^+ (Fig. 77). In this respect the flux data are not entirely suitable for a satisfactory full analysis, and this was borne in mind when interpreting the results. However, no large net gain (or loss) of any of the ions under study was occurring that would distort the outcome of the analysis unacceptably.

Tables 21 to 23 show the primary data obtained for each ion. Table 21 gives the apparent contents, efflux constants and half-times of exchange for each compartment for K^+. Apparent influx to the vacuole is also given. 4 sets of results are shown, control plantlets, plantlets incubated for 1 hour before elution with ABA, plantlets incubated for 18 hours before elution with ABA and fully mature turions.

Tables 22 and 23 give the results for Na^+ and Cl^- respectively, in the same manner as the K^+ data. Unidirectional fluxes and actual contents of the vacuole and cytoplasm, calculated using the data in tables 21, 22 and 23 and equations (1) to (5), are given in tables 24, 25, and 26. Concentrations and membrane fluxes were calculated from estimates of the compartment volumes and membrane areas and are shown in table 27.

From the K^+ data it can be seen that the concentration of K^+ in the cytoplasm is much lower in the turion relative to the control

Figure 76

Apparent uptake of K^+ from labelled 1X solution as a function of time (left-hand ordinate) - - - - . Net uptake of K^+ from labelled 1X solution as a function of time (right-hand ordinate) ———— .

● control fronds and ○ turions of S. polyrrhiza (N).

FIGURE 76

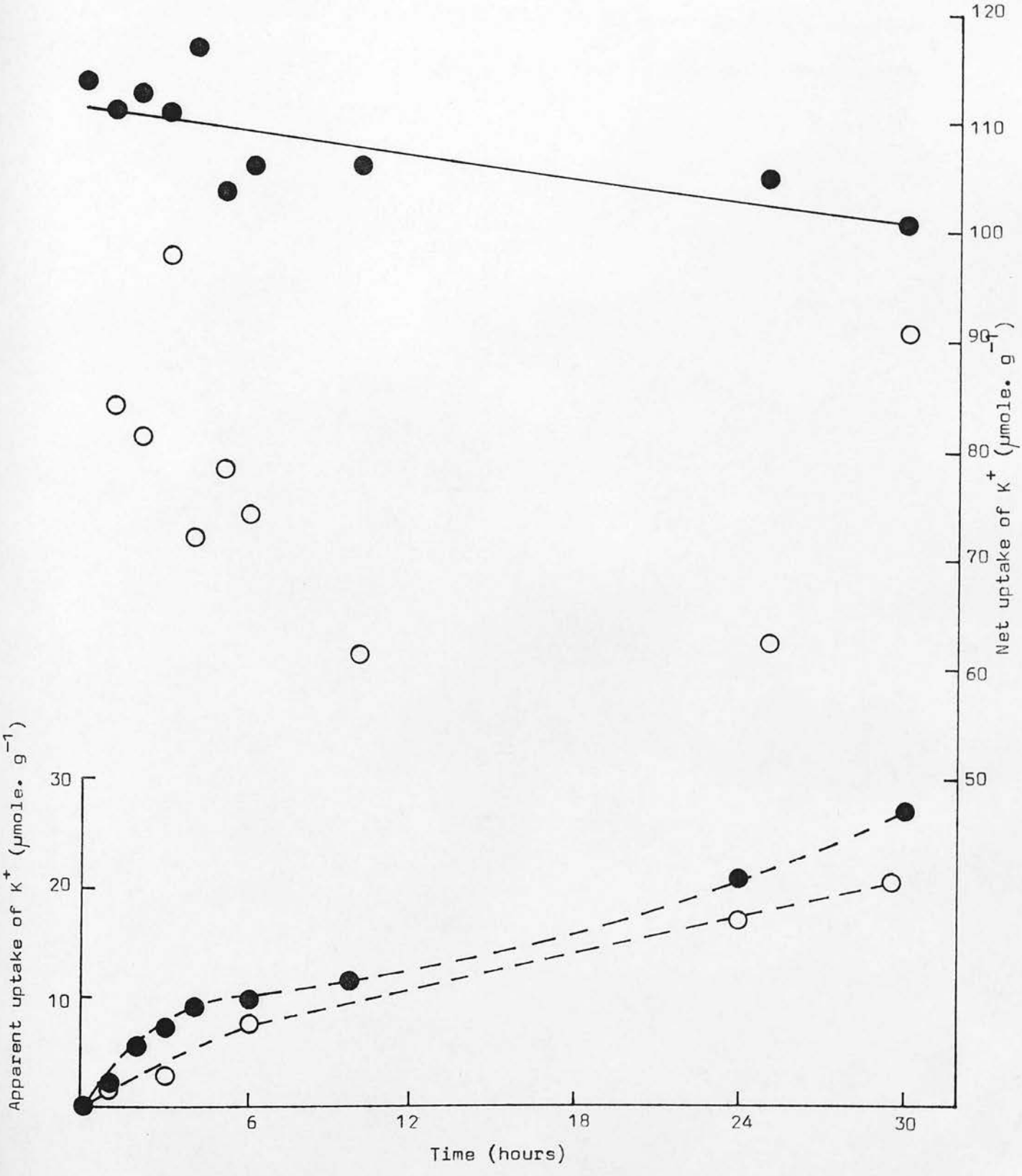

120
110
100
90
80
70
60
50
Net uptake of K+ (µmole. g-1)
30
20
10
Apparent uptake of K+ (µmole. g-1)
6
12
18
24
30
Time (hours)

Figure 77

Apparent uptake (left-hand ordinate)- - - -and net uptake (right-hand ordinate)——of Na^+ from labelled 1X solution as a function of time.

● control fronds and ○ turions of S. polyrrhiza (N).

FIGURE 77

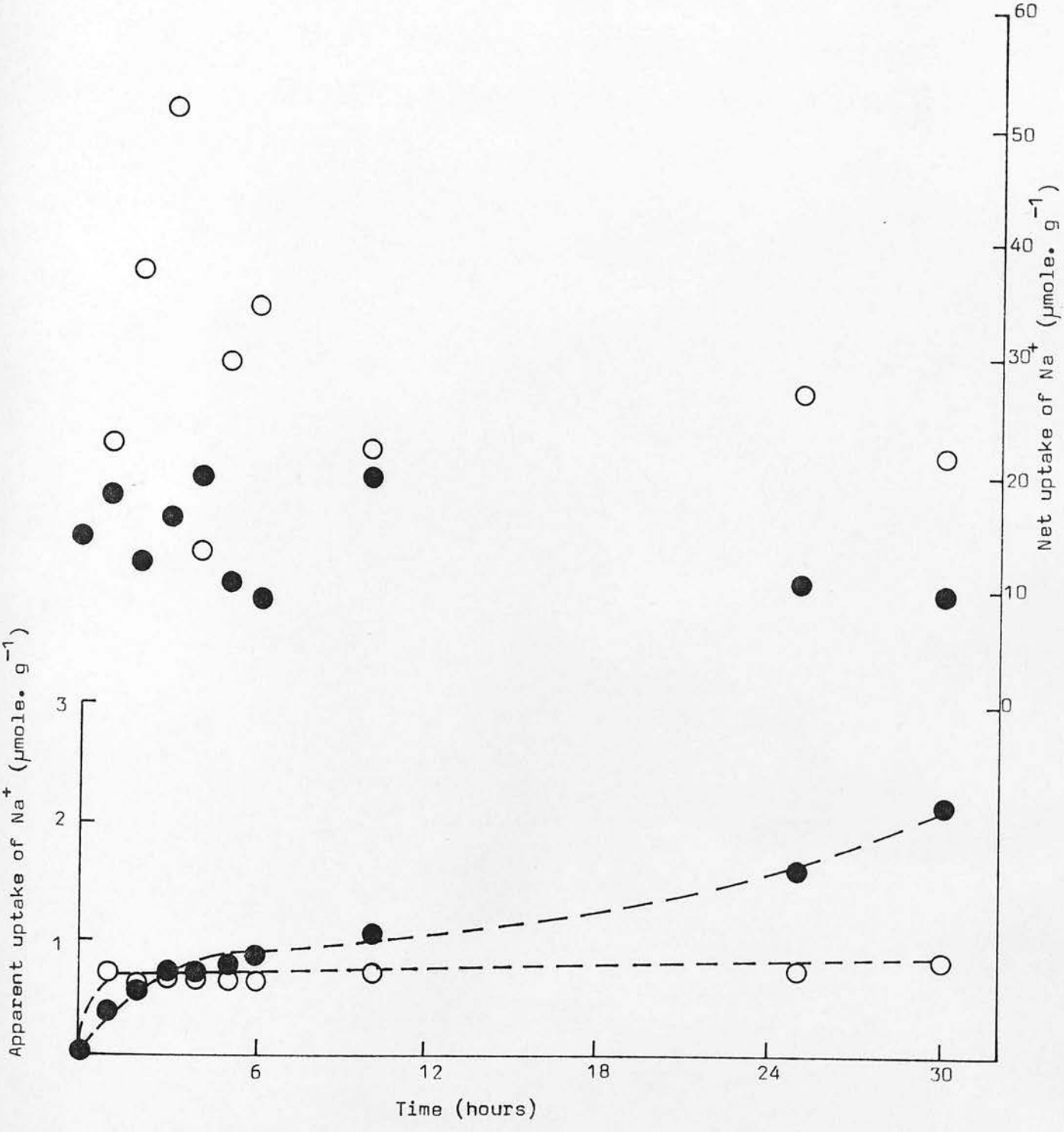

Apparent uptake of Na^+ (μmole. g^{-1})
Net uptake of Na^+ (μmole. g^{-1})
Time (hours)
0
10
20
30
40
50
60
1
2
3
6
12
18
24
30

Figure 78

Apparent uptake (left-hand ordinate)- - -and net uptake (right-hand ordinate)———of Cl^- from labelled 1X solution as a function of time. ● control fronds and ○ turions of S. polyrrhiza (N).

FIGURE 78

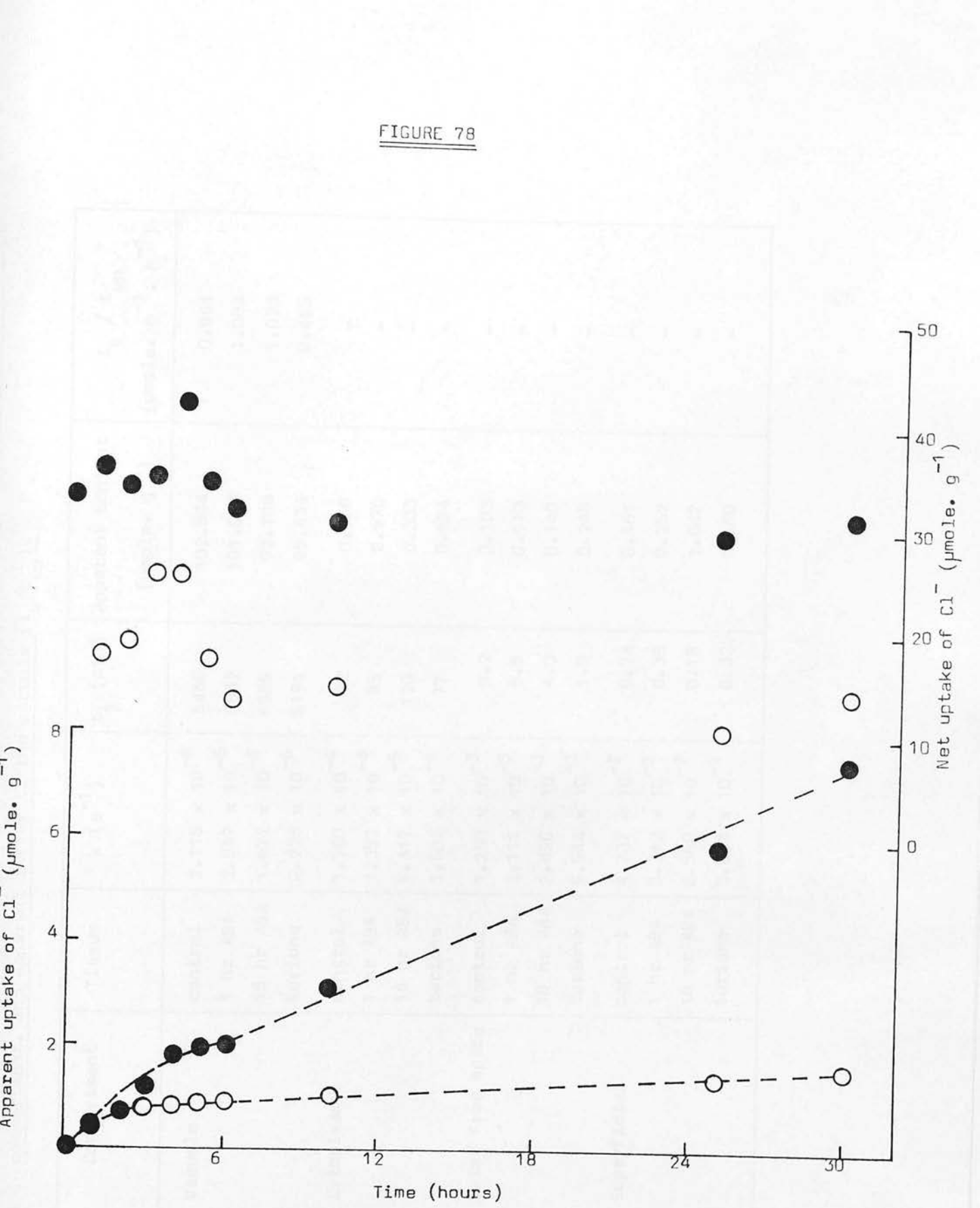

Apparent uptake of Cl^- (μmole. g^{-1})
Net uptake of Cl^- (μmole. g^{-1})
Time (hours)
2
4
6
8
0
10
20
30
40
50
6
12
18
24
30

Table 21: Efflux constants (k), half-times for exchange ($t_{\frac{1}{2}}$) and apparent contents for K^+ in each compartment, and apparent influx to the vacuole (I_v/t_{up}).

Compartment	Tissue	k (s^{-1})	$t_{\frac{1}{2}}$ (min)	Apparent content ($\mu mole.\ g^{-1}$)	I_v / t_{up} ($\mu mole.\ g^{-1}.\ h^{-1}$)
Vacuole	control	2.115×10^{-6}	5460	102.594	0.961
	1 hr ABA	2.047×10^{-6}	5642	101.009	1.084
	18 hr ABA	1.672×10^{-6}	6909	97.708	1.023
	turions	2.225×10^{-6}	5191	95.434	0.465
Cytoplasm	control	1.260×10^{-4}	92	0.568	-
	1 hr ABA	1.357×10^{-4}	85	0.570	-
	18 hr ABA	9.617×10^{-5}	120	0.333	-
	turions	1.503×10^{-4}	77	0.694	-
Water free space	control	1.250×10^{-3}	9.2	0.103	-
	1 hr ABA	2.111×10^{-3}	5.5	0.113	-
	18 hr ABA	2.890×10^{-3}	4.0	0.140	-
	turions	5.944×10^{-3}	1.9	0.248	-
Superficial	control	4.737×10^{-2}	0.24	0.461	-
	1 hr ABA	3.342×10^{-2}	0.35	0.252	-
	18 hr ABA	6.560×10^{-2}	0.18	1.047	-
	turions	3.653×10^{-2}	0.32	0.580	-

Table 22: Efflux constants (k), half-times for exchange ($t_{\frac{1}{2}}$) and apparent contents for Na^+ in each compartment, and apparent influx to the vacuole (I_v/t_{up}).

Compartment	Tissue	k (s^{-1})	$t_{\frac{1}{2}}$ (min)	Apparent content (μmole. g^{-1})	I_v / t_{up} (μmole. g^{-1}. h^{-1})
Vacuole	control	2.530×10^{-6}	4564	9.877	0.057
	1 hr ABA	3.184×10^{-6}	3627	7.582	0.041
	18 hr ABA	2.801×10^{-6}	4124	12.291	0.031
	turions	1.430×10^{-5}	808	26.954	0.006
Cytoplasm	control	4.264×10^{-4}	27	0.193	-
	1 hr ABA	4.264×10^{-4}	27	0.214	-
	18 hr ABA	3.325×10^{-4}	35	0.191	-
	turions	1.410×10^{-4}	82	0.083	-
Water free space	control	2.382×10^{-3}	4.9	0.102	-
	1 hr ABA	3.117×10^{-3}	3.7	0.117	-
	18 hr ABA	2.448×10^{-3}	4.7	0.111	-
	turions	4.060×10^{-3}	2.9	0.310	-
Superficial	control	6.736×10^{-2}	0.17	3.046	-
	1 hr ABA	6.954×10^{-2}	0.17	3.012	-
	18 hr ABA	6.807×10^{-2}	0.17	2.681	-
	turions	2.886×10^{-2}	0.40	0.431	-

Table 23: Efflux constants (k), half-times for exchange ($t_{\frac{1}{2}}$) and apparent contents for Cl^- in each compartment, and apparent influx to the vacuole (I_v/t_{up}).

Compartment	Tissue	k (s^{-1})	$t_{\frac{1}{2}}$ (min)	Apparent content ($\mu mole.\ g^{-1}$)	I_v / t_{up} ($\mu mole.\ g^{-1}.\ h^{-1}$)
Vacuole	control	2.061×10^{-6}	5603	22.912	0.226
	1 hr ABA	2.765×10^{-6}	4177	18.200	0.211
	18 hr ABA	2.921×10^{-6}	3954	19.044	0.234
	turions	1.621×10^{-5}	712	1.401	0.056
Cytoplasm	control	1.297×10^{-4}	89	0.100	-
	1 hr ABA	1.613×10^{-4}	72	0.049	-
	18 hr ABA	1.937×10^{-4}	60	0.073	-
	turions	2.709×10^{-4}	43	0.529	-
Water free space	control	2.513×10^{-3}	4.6	0.101	-
	1 hr ABA	3.200×10^{-3}	3.6	0.056	-
	18 hr ABA	4.245×10^{-3}	2.7	0.029	-
	turions	-	-	-	-
Superficial	control	7.453×10^{-2}	0.16	2.100	-
	1 hr ABA	7.693×10^{-2}	0.15	2.849	-
	18 hr ABA	7.598×10^{-2}	0.15	2.224	-
	turions	1.409×10^{-1}	0.08	1.384	-

Table 24: Unidirectional fluxes and contents of K^+ computed from the results in Table 21.

Compartment	Tissue	Influx (μmole. g^{-1}. h^{-1})	Efflux (μmole. g^{-1}. h^{-1})	Flux ratio	Content (μmole. g^{-1})
Vacuole	control	3.572	3.311	1.08	91.927
	1 hr ABA	3.684	3.268	1.13	90.639
	18 hr ABA	5.123	4.563	1.12	76.711
	turions	1.378	1.650	0.84	91.962
Cytoplasm	control	1.219	0.957	1.27	11.235
	1 hr ABA	1.362	0.946	1.44	10.940
	18 hr ABA	1.139	0.577	1.97	21.330
	turions	0.841	1.112	0.76	4.166

Table 25: Unidirectional fluxes and contents of Na^+ computed from the results in Table 22.

Compartment	Tissue	Influx (μmole. g^{-1}. h^{-1})	Efflux (μmole. g^{-1}. h^{-1})	Flux ratio	Content (μmole. g^{-1})
Vacuole	control	0.074	0.106	0.70	9.773
	1 hr ABA	0.052	0.097	0.54	7.521
	18 hr ABA	0.048	0.140	0.34	12.225
	turions	0.193	1.553	0.12	26.558
Cytoplasm	control	0.353	0.385	0.92	0.297
	1 hr ABA	0.370	0.415	0.89	0.275
	18 hr ABA	0.260	0.352	0.74	0.257
	turions	0.048	1.409	0.03	0.479

Table 26: Unidirectional fluxes and contents of Cl^- computed from the results in Table 23.

Compartment	Tissue	Influx (μmole. g^{-1}. h^{-1})	Efflux (μmole. g^{-1}. h^{-1})	Flux ratio	Content (μmole. g^{-1})
Vacuole	control	0.950	0.874	1.09	20.184
	1 hr ABA	1.329	1.269	1.05	15.164
	18 hr ABA	1.057	1.003	1.05	17.055
	turions	0.064	0.083	0.77	1.277
Cytoplasm	control	0.272	0.196	1.39	2.828
	1 hr ABA	0.239	0.179	1.34	3.085
	18 hr ABA	0.285	0.230	1.24	2.062
	turions	0.572	0.590	0.97	0.653

Table 27: Chemical concentrations in the cytoplasm and vacuole and fluxes across the plasmalemma and tonoplast of K^+, Na^+ and Cl^-.

Ion and Membrane	Tissue	Influx (μmole. cm^{-2}. s^{-1})	Efflux (μmole. cm^{-2}. s^{-1})	Concentration (mM)
K^+ tonoplast	control	5.728	5.309	188.14
	1 hr ABA	5.908	5.240	185.51
	18 hr ABA	8.215	7.317	157.00
	turions	1.820	2.179	246.81
K^+ plasmalemma	control	1.727	1.356	61.09
	1 hr ABA	1.610	1.340	59.49
	18 hr ABA	1.614	0.817	115.99
	turions	1.055	1.395	20.87
Na^+ tonoplast	control	0.119	0.170	20.00
	1 hr ABA	0.083	0.156	15.39
	18 hr ABA	0.077	0.224	25.02
	turions	0.255	2.051	71.28
Na^+ plasmalemma	control	0.500	0.545	1.62
	1 hr ABA	0.524	0.588	1.50
	18 hr ABA	0.368	0.499	1.40
	turions	0.060	1.768	2.40
Cl^- tonoplast	control	1.523	1.402	41.31
	1 hr ABA	2.131	2.035	31.04
	18 hr ABA	1.695	1.608	34.91
	turions	0.085	0.110	3.43
Cl^- plasmalemma	control	0.385	0.278	15.38
	1 hr ABA	0.339	0.254	16.78
	18 hr ABA	0.404	0.326	11.21
	turions	0.718	0.740	3.27

The results from Tables 24, 25 and 26 were corrected for membrane areas and compartment volumes using the data obtained by stereological analysis.

fronds. The turion plasmalemma and tonoplast flux ratios are both less than 1, which might be due to steady state conditions not being reached in the turions due to the change from H/2 (5·5 mM) to 1X (1 mM). However, this effect is also found with dormant storage tissue (van Steveninck, 1975; Macklon, 1976) where a net uptake of ions is not found until the tissue is sliced, a phenomenon known as adaptive ageing.

The turion of S. polyrrhiza appears to have much in common with dormant storage tissue, which is known to be incapable of developing an absorption capacity until the onset of the metabolic upsurge associated with slicing. This onset of net absorption is considered to depend on the progressive reduction of an initially high efflux. The development of the turion may involve the progressive loss of uptake capacity perhaps through an increase in the permeability of the plasmalemma for K^+ and an inefficient uptake mechanism. Interestingly however, treatment of fronds with ABA for 18 hours resulted in a reduced efflux relative to control plantlets, and the cytoplasm concentration increased. There was no rapid effect of ABA on the fluxes through either membrane.

Turion vacuolar concentration of Cl^- is very low compared with control fronds, and exchange across the tonoplast is also very low, most likely since the higher exchange rate across the tonoplast in the control fronds would have allowed higher levels of Cl^- to be achieved during the loading period. In the cytoplasm, even though the exchange rate in the turion is higher than in the control fronds, Cl^- concentration is still lower in the turion and efflux exceeds influx, as for K^+. There was no effect of ABA on the fronds.

The results for Na^+ are extremely puzzling. All effluxes are greater than influxes. While perhaps this was not surprising for tissue transferred from H/2 (0•1 mM Na^+) to 1X (1 mM Na^+), similar results were obtained in an experiment performed throughout in H/2 medium where the steady state should not have been affected (results not presented).

When considering the ion flux data as a whole, it is apparent that while the turion is very different from control plantlets with respect to its ionic properties, there is little effect of ABA on the fronds. The one example of an effect of ABA on the plantlets was for K^+ fluxes at the level of the plasmalemma, but this effect was only after 18 hours incubation in ABA, and the effect was in fact in the opposite direction to the situation in the fully developed turion.

Furthermore, preliminary experiments with the K^+ ionophore valinomycin and the Ca^{2+} ionophore A23187, showed that turion formation was neither induced by these ionophores alone, nor in conjunction with 10^{-8}M or 10^{-5}M ABA; and turion formation induced by 10^{-7}M ABA was unaffected.

CHAPTER 7

FINAL DISCUSSION

7.1 CRITICISM OF WORK

While the turion of Spirodela polyrrhiza is, by virtue of its small size, its ease of production by ABA, and its accessibility to be studied under sterile conditions, ideal for the study of dormant resting buds, several problems remain.

In an ecological sense the term 'dormancy' is usually applied to a phase in the life of a plant which permits it to survive a period of potential stress by virtue of an almost complete cessation of growth and an overall reduction in general metabolic activity. This condition is often preceded by various morphological changes which provide a generally increased resistance to adverse environmental conditions. In the vegetative phase such changes include leaf abscission, abortion of terminal growing points, the formation of underground storage organs with an increased resistance of individual cells to damage by freezing or desiccation, as well as the formation of dormant buds.

In some cases the dormant state is caused directly by the unfavourable external conditions (Vegis, 1964), which is usually referred to as 'imposed' dormancy. However, in many cases unfavourable conditions are not the direct cause of dormancy. Thus many trees form winter resting buds during the summer and autumn (as does S. polyrrhiza (Jacobs, 1947)), when temperatures and light conditions are evidently still favourable, and long in advance of the winter. In such plants the cause of dormancy appears to lie within the tissues of the buds themselves, the buds thus being 'innately' dormant.

Whether the turion of S. polyrrhiza which is induced by abscisic

acid is an innately dormant bud is a matter for argument. Certainly by the criteria outlined above, the turion remains dormant under environmental conditions otherwise suitable for growth (since these parameters are carefully controlled). However, the dormancy of the turion, although not as defined an imposed dormancy, is totally imposed by exogenously supplied ABA, and may bear little relationship to the dormant buds of woody perennials found in nature, or indeed even to naturally occurring turions of S. polyrrhiza.

Clearly it may be a gross oversimplification to suggest that bud dormancy is necessarily due to the presence or absence of a 'dormancy inducing growth regulator' or even to a specific balance between growth promotors and inhibitors, although the production of turions in S. polyrrhiza is perhaps the most convincing evidence suggesting that ABA plays an important role in dormancy (Stewart, 1969; Perry and Byrne, 1969; Saks et al., 1980).

Another difficulty with the system studied here is the lack of a truely satisfactory control. When ABA is added to a culture of S. polyrrhiza, turions are produced. In measuring the biochemical events associated with turion formation during the development of the turion from a small bud less than 0·7 mm long to the fully developed turion of 2 mm, certain trends became apparent e.g. a decline in DNA, protein and RNA synthesis, elevation of anthocyanin and starch levels. Throughout this work the comparison has been made between primordia which have the ability to form turions following the application of ABA, and the fully developed turions formed several days later. Is this really a meaningful internal control, or should the turion be directly compared with the mature

mother frond, which is after all the destination of the primordium in the absence of ABA?

The situation is not even as simple as this, for in normal development the primordium would by that time have produced at least two daughter fronds i.e. would have developed into a plantlet. However, in terms of size the turion is only 2 mm long, and perhaps the development of the turion should be followed by a parallel study on the development of a vegetative primordium to a vegetative frond of 2 mm in length. The time scale of events in both processes would make this extremely difficult. The possibilities of comparison become virtually endless, and so a compromise had to be made. In the majority of experiments the developing turion was compared during its development to the primordium before ABA addition, although in many experiments the biochemical parameter was also followed during the development of the vegetative frond, and in the plantlets which remained after removal of the developing turions each day after ABA addition i.e. the turion producing mother fronds.

It is also appreciated that the general method of analysis is not without its drawbacks. Each day after ABA addition, developing turions were dissected from the plantlets. Although the average size and therefore the developmental age of the developing turions increases with the time of incubation in ABA, the developmental stages dissected on one day obviously varied in size. This was more noticeable during the latter part of turion formation, when the whole range of developmental stages would have been present. To overcome this problem would require the dissection of specific developmental stages over a long period of time, and storage until

sufficient material had been accumulated. Alternatively, and perhaps more appealing, would be the development of micro-scale assay techniques throughout, which would enable individual fronds or developing turions to be studied in isolation (e.g. Neuhof, 1973; Jones et al., 1977; Jones, 1979). The mother fronds associated with the developing turions also contain many small primordia within their meristematic pockets which could not be easily removed. The effect of ABA on isolated mother frond tissue could possibly be investigated by using the distal half of the frond only, a technique which has been adopted by Weinbaum et al. (1979) in their study of the 32,000 dalton chloroplast membrane protein in Spirodela oligorrhiza.

The turion of S. polyrrhiza was chosen for this work, not only in order to understand more about turion formation and possibly the development of resting buds in general, but since it is inducible by ABA, to investigate the role of ABA in this process, and therefore the mode of action of ABA in general. For convenience, terminology such as "the effect of ABA on the developing turion or mother frond" has been used throughout this work. This would imply that the changes observed are solely due to the effect of ABA on a particular developmental programme which is already in progress. However, ABA induces the developmental programme and is not simply affecting the status quo. This criticism may not apply to the effect of ABA on the mature tissue, since it is not itself undergoing the turion developmental programme, although it should not be assumed that the mother frond plays no part in turion formation, since it is in the meristematic pockets of the mother frond that the stimulus for

turion formation must be initiated. One tool which would enable us to establish those effects of ABA which are specific to the process of turion formation would be a mutant of S. polyrrhiza which, while responding to ABA with respect to growth inhibition, would never give rise to turions under any circumstance. A mutant of S. polyrrhiza which never seems to produce turions has been reported (Perry, 1968; Perry and Byrne, 1969), although all attempts by myself to obtain this mutant have failed. Unfortunately (and interestingly) this mutant does not respond to ABA even with respect to growth inhibition.

A considerable amount of literature has given us few definite answers about the role of ABA in regulating plant processes, especially dormancy. A role for ABA in the induction and maintenance of bud dormancy has been neither unequivocally demonstrated nor disproved. The major barrier to demonstrating such a role is that the precise nature of the biochemical events leading to or from dormancy are not understood and we are unable to determine whether or how ABA can influence these events. Furthermore, although we now have considerable information about total ABA levels in tissues and how they are affected by various environmental treatments, we are almost completely ignorant about its cellular localisation, compartmentation, and the regulation of its synthesis and movement into the tissues involved.

The induction of turion formation by ABA in S. polyrrhiza is potentially an extremely useful system, since it is one of the few examples where ABA clearly induces a morphogenesis into the dormant state. The approach taken in this work was, of necessity broad,

since it was hoped to characterise the system as fully as possible, and therefore eventually to gain an insight into the processes inherent in the formation of dormant buds.

7.2 IS INHIBITION OF GROWTH NECESSARY FOR TURION FORMATION?

Addition of ABA to a culture of Spirodela polyrrhiza resulted in growth inhibition at concentrations as low as 10^{-9}M, growth of the culture being completely arrested at 10^{-5}M. A plot of growth inhibition against the log of the concentration of ABA was found to be sigmoid (a behaviour typical of the response of a growing plant to ABA (Milborrow, 1978)).

In discussing the concentration range over which ABA is effective, one should note that whilst the inhibition curve for ABA spans a 10^4 fold concentration range, the majority of growth inhibition occurs over a much narrower range. Thus between 10^{-8}M and 10^{-6}M ABA, growth inhibition increases from 14% to approximately 80% (~5½ fold change). Even so, the extent of change is no greater than the degree of response of other systems to ABA (Milborrow, 1978) and indeed is consistent with information on other plant growth regulators (Trewavas, 1981). A response graduated over such a range of concentrations is arguably not consistent with a rapid cascade or amplification type system so typical of the response of mammalian tissue to a given hormone or transmitter (Cuatrecasas, 1974).

However, over a single order of magnitude range around 10^{-7}M, ABA additionally induces turion formation in S. polyrrhiza, which is reputably an atypical response for a plant growth regulator

(Trewavas, 1981). It should be noted however, that Stewart (1969) claims to have obtained turions over a much wider ABA concentration range (see section 3.2.1).

Growth inhibition in the Lemnaceae by ABA is extremely potent and can be reversed at any time, following removal of ABA (Stewart, 1969; van Overbeek et al., 1967; McLaren and Smith, 1976). My work has shown that the concentration of ABA for turion formation is critical. Although it is quite conceivable that a threshold level of ABA is required to initiate the response, the lack of response at high ABA levels does not necessarily indicate an upper limit for the initiation of the response, since the growth inhibition response at these high levels of ABA may prevent the molecular and biochemical steps leading to turion formation.

It is evident that where ABA effects turion formation, some attendant inhibition of frond growth results. Whether this inhibition is an essential prerequisite of turion formation, or merely represents an additional dose correlated action of ABA and is thus a separate and distinct response, is at present moot.

It is of interest to note the existence of the following evidence:

a) a large number of the Lemnaceae exhibit a dose related growth inhibition response to ABA without any concomitant turion formation (van Overbeek and Mason, 1968; van Staden and Bornman, 1970a; Chen and Park, 1976).

b) Stewart (1969) has shown that turion formation in S. polyrrhiza, albeit a small amount, can occur at concentrations of ABA which result in no growth inhibition.

This would appear to be strong evidence that growth inhibition is not a prerequisite of turion formation, and that a priori turion formation is a distinct response to ABA by S. polyrrhiza. However, this conclusion should be tempered by the following facts:

a) many other authors find that turion formation, induced by ABA (Perry and Byrne, 1969; Saks et al., 1980) or otherwise (Jacobs, 1947; Czopek, 1959) is always accompanied by substantial growth inhibition. Similarly naturally occurring turion formation is accompanied by frond growth inhibition (Stewart, 1969).

b) the existence of a mutant of S. polyrrhiza which shows neither inhibition of growth nor turion formation in response to ABA (Perry and Byrne, 1969).

With regard to this latter observation, it would be necessary to postulate that this mutant had somehow lost the genetic apparatus that was essential for two apparently unrelated systems of response to ABA. On further examination of Stewart's paper, there appears to be an almost mirror image relationship between the growth inhibition and turion formation curves over a 10^5 fold spectrum of ABA concentration. This might be construed as supportive evidence for the suggestion that growth inhibition and turion formation are intimately linked aspects of a single process.

While growth inhibition alone is not causal in turion formation (Stewart and Smith, 1972; McLaren and Smith, 1976), it is clear that ABA will only result in turion formation in the strain used in this work in combination with a specified level of growth inhibition. It is interesting that in S. polyrrhiza (N) turions are produced in response to ABA at concentrations resulting in a growth inhibition

of between 44% to 70%, with 48% inhibition at 10^{-7}M ABA, the optimum concentration for turion production. This compares very favourably with the observation that in nature turion production is accompanied by a 40% to 60% inhibition of growth (Stewart, 1969).

On balance it would seem expedient at present to assume that inhibition of growth is an integral and essential part of the turion forming response to ABA. It does not seem inherently unlikely that both responses could and do result from the action of ABA at a single locus (e.g. receptor or linked receptors).

The question of whether endogenous ABA inhibits normal growth and induces the formation of turions is difficult to answer since a specific disruption of the ABA biosynthetic pathways is at present not possible. Similarly, attempts at selectively blocking the action of endogenous or applied ABA must await the advent of a specific antagonist (possibly a structural look-alike). Perhaps the best evidence for a role of endogenous ABA in turion formation comes from the recent work of Saks et al. (1980). These authors showed that the medium in which a turion forming culture (induced by a short photoperiod) had been growing, inhibited growth and induced turions in a fresh culture (grown under a long photoperiod), and that the released factor was in all probability ABA. Moreover, the time of appearance of ABA in the medium and the rise in concentration during the following days correlated with the onset of the process of turion formation. The hypothesis that ABA is involved in the control of turion formation in S. polyrrhiza also finds support in the results of Weber and Nooden (1976) who worked with the aquatic plant Myriophyllum verticillatum and found an increase in the

internal level of acidic inhibitors which co-chromatographed with ABA at the time of turion appearance. However, in this species exogenously applied ABA did not induce turion formation under non-environmentally inductive conditions, but only enhanced turion formation induced by a short photoperiod.

Another property of growth inhibition by ABA indicating an endogenous role, is its reported reversal by certain cytokinins (van Overbeek et al., 1967; van Overbeek and Mason, 1968; van Staden and Bornman, 1970b). Reversal of natural and abscisic acid induced turion formation by cytokinins has been reported in S. polyrrhiza (Stewart, 1969; Saks et al., 1980), although reversal of ABA induced turion formation was paradoxically accompanied by increased growth inhibition (Stewart, 1969). It is clear that while growth inhibition by endogenous or exogenous ABA may be antagonised by cytokinins in some allosteric manner (van Overbeek et al., 1967), the interaction might just as conceivably be of a non-competitive nature.

7.3 SENSITIVITY TO ABA AND ITS VARIATION

One important feature of turion formation in S. polyrrhiza (N) was that only primordia below a certain developmental stage could be induced by ABA to develop into turions. It was found that the destiny of a particular primordium was determined by the time it reached approximately 0·7 mm in length i.e. one tenth of the size of the mature vegetative frond. All the cells of a primordium at this or a younger developmental stage were responsive to ABA in that they all developed into turion cells, and subsequently after a few days

growth into fully formed turions. The production of semi-turions from primordia at later developmental stages (0·8 mm - 1·3 mm) was indicative of a differential sensitivity to ABA throughout the development of the frond, and therefore presumably a differential cell sensitivity to ABA. It must be pointed out that the term 'sensitivity' as used here does not necessarily indicate an altered perception of a particular cell to ABA, but is simply a measure of the cell's ability to respond to ABA with respect to developing into a turion cell several days later.

A summary of the types of frond produced during turion formation is shown in figure 79a. Detailed analysis of individual frond growth curves (section 3.2.2) allow for the period of maximum sensitivity to ABA to be calculated (Fig. 79b) on the basis that the vegetative and turion primordia are morphologically indistinguishable until they are 0·45 mm long, and that the vegetative primordium is no longer able to develop into a turion once it is over 0·7 mm long.

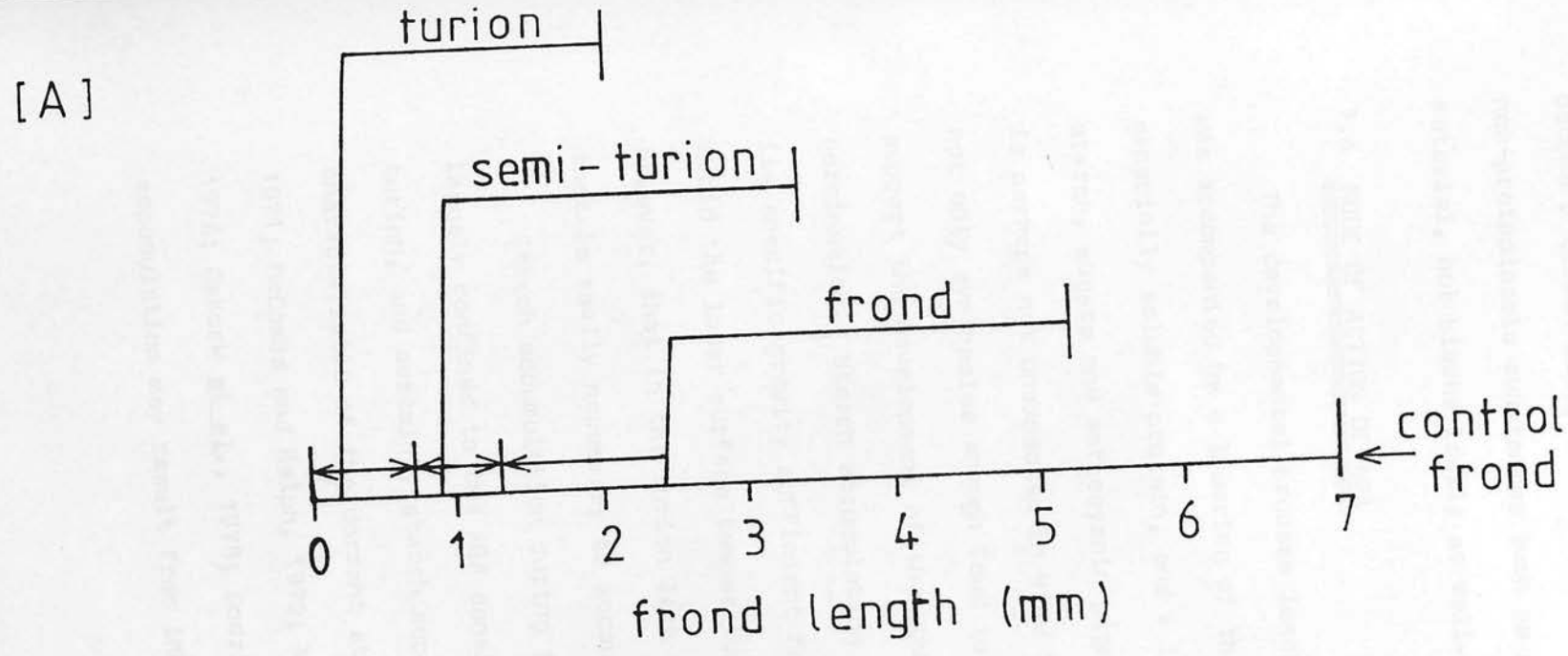
Thus it is postulated that during the development of a cell it acquires ABA sensitivity as far as turion formation is concerned and is only in this state for approximately 14 hours. Ultrastructural analysis showed that the cells within this sensitivity window are still actively dividing, and significant expansion of the individual cells has not yet occurred. As the frond approaches the end of the window i.e. 0·7 mm long, cell expansion is taking over from cell division as the main growth process at the distal end of the frond and the process of air space formation is just beginning. It is felt that this developmental switch-over to rapid cell expansion and

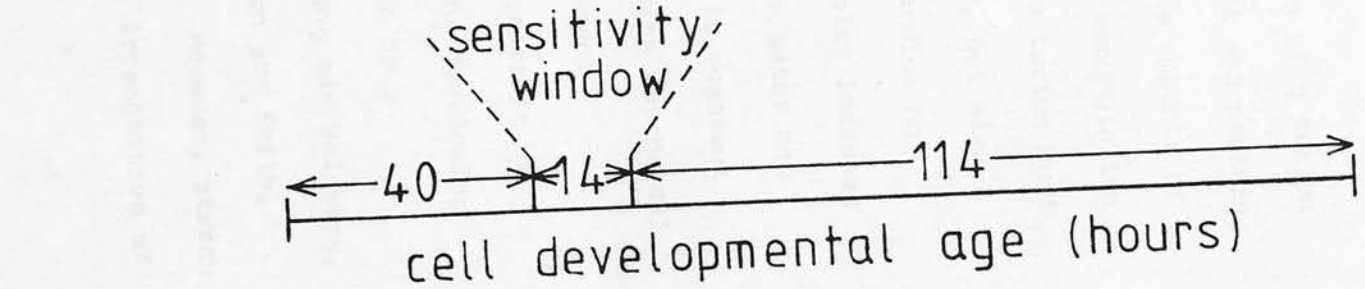
Figure 79

(a) A summary of the frond types produced during turion formation of S. polyrrhiza (N) in 1 x 10^{-7} M ABA. Fronds of 0·7 mm or less develop into turions which attain a length of 2 mm after 7 days; fronds longer than 0·7 mm and shorter than 1·3 mm develop into semi-turions which attain a length of 3·4 mm after 7 days; and fronds longer than 1·3 mm develop into vegetative fronds which seldom exceed 5·2 mm in length after 7 days. Control fronds (no ABA) often reach a length of 7 mm in the same time period.

(b) Detailed analysis of individual frond growth curves allow for the period of maximum sensitivity to ABA to be calculated. The sensitivity window is 14 hours wide and cells within this age range can respond to ABA by developing into a turion cell upon addition of ABA.

FIGURE 79


[A]
turion
semi-turion
frond
control
frond
0
1
2
3
4
5
6
7
frond length (mm)


[B]
sensitivity
window
40
14
114
cell developmental age (hours)

separation marks the end of the cell's sensitivity to ABA.

The differential sensitivity to ABA during the cell's development is not only seen in its ability to morphologically become a turion cell, characterised by extremely large deposits of non-protoplasmic substances such as starch, tannins, and cell wall material, but biochemically as well.

7.4 MODE OF ACTION OF ABA

The developmental process leading to the formation of a turion was accompanied by a lowering of the levels of DNA, RNA and protein, especially soluble protein, and a large increase in the amounts of starch, sugars and anthocyanin pigment. Carbohydrate accumulation is perhaps not unexpected in view of the fact that the turion must not only synthesise enough food to survive the winter, but also support the development of the young vegetative primordium on germination. Starch accumulation by the turion may also increase its specific gravity sufficient for it to sink in the water and avoid the lower surface temperatures in winter. It is apparent however, that in the turion lack of air space formation may be all that is really necessary to account for this phenomenon.

Starch accumulation during turion formation appears to be largely confined to the ABA sensitive tissue i.e. the developing turion, and certainly starch accumulation appears to be a characteristic of the dormant state (Tasseron-de Jong and Veldstra, 1971; McCombs and Ralph, 1972; Newton, 1974; McLaren and Smith, 1976; DeKock *et al.*, 1978; Godziemba-Czyz, 1969b). However, starch accumulation may result from inhibition of growth, irrespective of

how such inhibition is effected, and therefore this raises the question of whether ABA has a more direct or specific role in interfering with carbohydrate metabolism.

The observation that CO_2 fixation during photosynthesis is often reduced when starch accumulates in the chloroplasts (McLaren and Smith, 1976, 1977; Jensen, 1980) has lead to the hypothesis of product inhibition of photosynthesis. Endogenous sucrose appears to have little adverse effect on the photosynthetic rate, most likely because sucrose is not formed nor located inside the chloroplast (Bird et al., 1974). The drop in photosynthesis with high starch content in the chloroplast has been proposed to be due to the physical distortion of the chloroplast by accumulating starch grains, and this might certainly explain the reported decline in photosynthesis of the turion of S. polyrrhiza (Czopek, 1967), on the basis of my ultrastructural findings.

McLaren and Smith (1976) found that ABA decreased the permeability of the chloroplast envelope to glycerate 3-P. If ABA did have a direct effect on the movement of glycerate 3-P across the chloroplast, then possibly a build up of this metabolite inside the chloroplast would lead not only to increased starch synthesis (since ADP-glucose pyrophosphorylase is allosterically activated by glycerate 3-P; Steup et al., 1976), but also to decreased mobilisation. Starch stored in the chloroplast during the day is mobilised to soluble products (mostly glycerate 3-P and maltose) and exported from the chloroplast at night (Lilley et al., 1977). This mobilisation is inhibited by glycerate 3-P. McLaren (1976) proposes that ABA inhibits the transport of glycerate 3-P across the

chloroplast envelope by directly interacting with the phosphate translocator (Heldt, 1976).

Carbohydrate accumulation does not appear to be responsible for turion initiation, but is probably merely a result of ABA induced growth inhibition and therefore decreased utilisation of carbohydrates (McLaren and Smith, 1976). However, ABA does appear to be involved in a number of aspects of photosynthesis, including development of plastids (Wellburn et al., 1973), photosynthetic enzyme activity (Sankhla and Huber, 1974, 1975; Bauer et al., 1976; Ryć and Lewak, 1980), photochemical events (Bauer et al., 1976; Raghavendra et al., 1976) and starch and sucrose hydrolysis (Chrispeels and Varner, 1966; Saunders and Poulson, 1968). Not all processes are affected by ABA in all cases, and the stage of development of the cell seems to be an important factor in determining the nature of the response. In no case has the primary target of ABA in regulating photosynthesis been defined.

The differential sensitivity of cells to ABA with respect to regulation of photosynthesis and related processes is clearly highlighted by certain interesting anomalies found in this work.

Whereas the chlorophyll/carotenoid ratio of turion producing mother fronds remained unchanged in the presence of ABA as did the ratio in the turion during its development, the ratio of chlorophyll a/b in the mother fronds was increased during ABA incubation. Similarly, this effect was shown by McLaren and Smith (1976) with plantlets of Lemna minor. In both L. minor plantlets and the mother fronds of S. polyrrhiza the increase in the a/b ratio was mostly due to the decrease in the proportion of chlorophyll b. Since

chlorophyll b has been implicated in photosystem II (Jensen, 1980), the specific decline in chlorophyll b may indicate that ABA may affect this system. However, during the development of the turion, there is a decrease in the ratio, this time mostly due to an increase in chlorophyll b. Clearly if ABA does affect photosystem II (Bauer et al., 1976), the opposite action of ABA in the two tissues clearly represents such differential sensitivity.

Before leaving the question as to how ABA interferes with carbohydrate metabolism and what role it may play in turion formation, it may be relevant to recall that turion formation can also be induced by certain sugars (Henssen, 1954; Czopek, 1959; Newton et al., 1978). Such sugar induced turion formation is also accompanied by increased carbohydrate levels within the turion (Henssen, 1954). In view of the fact that ABA is reported to be bound in vivo as the glucosyl ester (Milborrow, 1974), sugar accumulation might be necessary to maintain physiologically active levels of ABA in the cytoplasm of the Lemnaceae, since the glucosyl ester of ABA is just as active as ABA on a molar basis (Koshimizu et al., 1968). It would be especially important for a plant suspended in an aquatic environment to complex ABA in some way, since the plasmalemma would probably present little resistance to the movement of ABA down its concentration gradient into the surrounding aqueous milieu (Weyers and Hillman, 1979). Presumably the plasmalemma would be less permeable to the very much more polar glucosyl ester of ABA than to ABA.

It is of interest that should the level of free glucose be in some way limiting relative to that of ABA, then any increase in the

level of the sugar may result in a corresponding increase in the level of the glycosylated derivative. In this way the level of ABA may increase to physiologically active levels and might then initiate the processes leading to turion formation. This might explain why past researchers (Jacobs, 1947; Czopek, 1963) have concluded that any condition resulting in high photosynthetic levels over and above that required for growth and thus respiration lead to turion formation. Consequently, exogenously supplied sugars could therefore result in a similar sequestering of ABA and subsequent expression of a turion forming response. Although 'bound' ABA has often been assigned an inactive or storage role in land plants (Wright, 1975), the level of bound ABA in growing plants is relatively small in comparison with free ABA (Zeevaart, 1971; Hiron, 1974), and the fact that the completely submerged hygrophyte Ceratophyllum demersum has very little free ABA (Milborrow and Robinson, 1973), could be seen as supportive evidence to the above speculation. Future work in this area could involve investigations into the temporal resolution of sugar accumulation in environmentally induced turions, and on the nature and activity of glycosylating enzymes during turion formation.

The levels of accumulated sugars might also explain the observed rise in anthocyanin pigment associated with the latter stages of turion formation, since sugar accumulation is most certainly required for pigment production in the Lemnaceae (Thimann and Edmondson, 1949). Interestingly the pigments synthesised during turion formation are characterised by a higher degree of glycosylation (Reznik and Menschick, 1969) than those formed in non-turion

forming cultures. However, whether anthocyanin accumulation is merely a direct result of increased sugar levels during turion formation or whether additionally ABA plays a more direct role in directing the synthesis of pigment is not known.

The enzyme phenylalanine ammonialyase (PAL) which occupies an important position as a potential regulator of phenolic biosynthesis in higher plants (Zucker, 1972; Camm and Towers, 1973; Phillips and Henshaw, 1977) has often been regarded as a potentially important regulator of pigment level. Although it is unlikely that PAL is the only regulatory enzyme in flavonoid biosynthesis, there is extensive evidence that PAL levels are regulated not only by phytochrome (Smith and Attridge,1970), but also by plant growth regulators (Haddon and Northcote, 1976) which leads to changes in pigment levels. PAL catalyses the deamination reactions in which phenylalanine and tyrosine are degraded respectively to trans-p-cinnamic and trans-p-coumaric acids plus ammonia. These reactions possibly represent an important metabolic sequence in plants, which results in the diversion of these two amino acids from the pathways of protein synthesis into secondary metabolism, with the subsequent production of metabolites such as lignins, flavonoids, tannins and alkaloids (Smith, 1975). Thus PAL has a pivotal role in the reactions of secondary metabolism and in this manner may exert its influence on plant growth and development. It would be of interest to investigate PAL activity during the development of the turion, and the possible regulation of enzyme activity by ABA, since turion formation is a system which is characterised by reduced synthesis of proteins and increased anthocyanin production.

Although secondary metabolism appears to be enhanced during the development of the turion, there appeared to be drastic repression of nucleic acid and protein synthesis. The development of the vegetative Spirodela frond involves the processes of cell division, expansion, differentiation and separation. Since ABA induced the formation of turions with concomitant inhibition of growth, it seems possible that the mode of action of ABA might involve interference with DNA replication involved in cell division and DNA-dependent RNA synthesis, since differentiation must involve changes in the protein complement of the cell.

The first change observed during turion formation was a striking inhibition of DNA synthesis in the developing turion within 3 hours of ABA application. This is entirely consistent with reports of inhibition of DNA synthesis in the Lemnaceae, albeit with higher concentrations of ABA (van Overbeek et al., 1967; Stewart and Smith, 1972). Turion inducing concentrations of ABA have very little effect however on the rate of DNA synthesis in the majority of the tissue; which has a relatively low rate of synthesis probably due to the inherent low rate of cell division. While the possibility of a direct effect of ABA on the synthetic mechanism involved in DNA replication cannot be ruled out, any such effect is unlikely to be causal in turion formation since to date turion formation has not been induced by alternative inhibitors of DNA synthesis (Stewart and Smith, 1972). However, as far as is known, combinations of such inhibitors with ABA have not been tried, and would be an interesting area for further work.

The evidence against a role for DNA synthesis in ABA action

comes mainly from systems where there is no cell division (Haber et al., 1969; Paranjothy and Wareing, 1971), although the limits of DNA detection in the assays used have been questioned (Taiz, 1975). Perhaps the best evidence against DNA synthesis being the primary target of ABA comes from the work of Chen and Osborne (1970), who found a sequential inhibition of protein synthesis before RNA synthesis, with DNA synthesis being affected last of all in dry wheat embryos. It is possible however, that in systems involving cell division, ABA may have a primary effect on the mechanism of cell division, thus leading to an inhibition of DNA synthesis.

However, in contrast to the effect of ABA on whole plantlets of S. polyrrhiza (Stewart and Smith, 1972), there was found no sequential inhibition of DNA, RNA and protein synthesis in the developing turion of S. polyrrhiza (N). Protein synthesis was inhibited before that of RNA, which was not measurably affected until 3 days after ABA application. Moreover, the inhibitory effect of ABA on protein synthesis during the development of the turion appeared to be selective, as shown initially by a preferential decline in the level of soluble protein, and later by an investigation of the in vivo changes in the proteins synthesised each day after ABA application. In other words, although the rate of total protein synthesis was inhibited, the rate of decline varied between individual species. There was also an increase in the synthesis of certain proteins compared to the overall pattern and several novel proteins appeared during turion formation. Blanket inhibition of protein synthesis by azetidine 2-carboxylic acid, did not induce turion formation either alone or in combination with low levels of

ABA.

A major question in dormancy is the degree of involvement of transcriptional and translational control of enzyme production. Although in several other systems, ABA is thought to act via an overall inhibition of RNA synthesis (van Overbeek et al., 1967; Poulson and Beevers, 1970; Stewart and Smith, 1972; Walbot et al., 1975) this is clearly not the case during ABA induced turion formation. The inhibition of RNA synthesis is not significant until 3 days after ABA application, when the biochemical events leading to the production of the turion cell are virtually complete.

The rapid general inhibition of protein synthesis at early stages of turion formation could not be accounted for by changes in the total level of translatable mRNA. The specific alterations to the pattern of proteins labelled in vivo could however have resulted from control of the level of translatable mRNA for these particular species, since changes in the mRNA population are noticeable within 24 hours of ABA application (earlier times were not studied). It is also quite feasible that some of the new proteins synthesised early in turion formation, perhaps arising from de novo mRNA synthesis, might be involved in regulating the translation of existing mRNAs, thus leading to a decline in the synthesis of many other proteins, including perhaps those necessary in cell division. It can be seen therefore that although the general decline in protein synthesis precedes the decline in the mRNA population, a direct effect of ABA at the translational or post-translational level need not necessarily be inferred.

Studies involving the use of a much simpler system, the barley aleurone, have not conclusively distinguished between an effect of ABA at the transcriptional or post-transcriptional level. Jacobsen et al. (1980) suggest that ABA inhibits gibberellin induced α-amylase synthesis by inhibiting the accumulation of translatable mRNA which could involve transcription, mRNA modification, or degradation of the mRNA. Conversely Ho and Varner (1976) found evidence that the inhibitory action of ABA is due, at least in part, to an effect on translation. More recent evidence (Mozer, 1980) confirms the findings of Ho's group, namely that ABA controls the production of α-amylase by effectively blocking the translation of the mRNA in vivo.

Both views have obtained support from work carried out in other systems. Transcriptional changes involved in the action of ABA are strongly suggested by the effects of ABA on in vitro transcription in radish hypocotyl and pear embryos (Pearson and Wareing, 1969; Khan, 1972) and polymerase activity in maize coleoptiles (Bex, 1972b); while evidence from studies on cotton embryos (Ihle and Dure, 1970) and sugar cane discs (Gayler and Glasziou, 1969) is consistent with the notion that ABA regulates post-transcriptional events.

While controversy remains between the various workers on the barley aleurone, all three groups have observed that ABA treatment of aleurone layers causes the appearance of several new proteins in vivo and when aleurone mRNA was translated in cell free systems (Ho, 1979; Jacobsen et al., 1980; Mozer, 1980). These findings along with my own observations on the developing turion, indicate that the

mode of action of ABA is not merely that of a non-specific inhibitor or antagonist of GA_3. Indeed, if it were, it would be difficult to envisage how it would cause the complicated switching events leading to morphogenesis in Spirodela.

The appearance of new proteins and new mRNA species during ABA induced turion formation raises the question as to the mechanism of action of ABA in this system. In addition, what role these proteins have in the latter processes of a more or less complete shut-down of all primary synthetic processes as the turion approaches maturity, remains to be answered.

7.5 MECHANISM OF ACTION OF ABA

In determining how ABA induces turion formation, it is essential to distinguish between the initial trigger reaction (or mechanism of action), and the series of steps which lead to turion production (or mode of action). Thus it can be hypothesised that the mechanism of action of ABA in responsive tissue is the regulation of gene expression and the production of specific mRNAs; followed by a sequence of steps leading to inhibition of DNA, protein and RNA synthesis, inhibition of cell division, expansion and differentiation, and an enhancement of secondary metabolism and carbohydrate accumulation.

Clearly however, it is a very difficult task to attempt elucidation of the details of the mechanism of ABA action in the control of a process such as turion formation, which involves so many growth processes. In attempting to elucidate the mechanism of action we need to solve two basic questions. The first is: does the

great diversity of responses reflect one or multiple mechanism(s) of action and what is the nature of the cellular component(s) which perceive ABA? The second is: what is the physiological state of the cells being exposed to ABA? The genes being expressed at the time of exposure clearly play an important role in how each cell responds. Multiple perceptors allow considerably more flexibility in the ways in which ABA might induce turion formation, since diverse responses are envisaged as arising from independent primary interactions of ABA with the cell; although this probably introduces more complexity than is required to explain ABA action.

There has been considerable debate during recent years as to whether the rapid action of ABA in some cases (stomatal closure, ion transport), which appears to be primarily an effect on cell membranes (Mansfield and Jones, 1971; Reed and Bonner, 1974), can account for the effects on protein synthesis and other responses which occur more slowly.

Plant growth regulators could exert an effect on membrane transport in three main ways by:

a) regulating enzymes,

b) acting directly on membranes,

c) affecting the availability of metabolic energy for ion transport.

The first possibility has long dominated as an explanation of the molecular level of control of plant growth regulators in general. The various ways in which plant growth regulators may control enzymes has been excellently reviewed by Jacobsen and Higgins (1978a, 1978b). These include de novo synthesis of enzymes via operation of the genetic code (transcription, translation),

enzyme degradation and various types of enzyme inactivation. Membrane and transport effects could be explained by alterations of membrane constituents through enzyme activity, or as secondary consequences of altered uptake and redistribution of materials.

It is assumed that a certain amount of time, of the order of many minutes up to one or two hours, is required for a complicated regulation via the genetic code. Therefore there has been a search for fast responses to support the idea of a primary action of plant growth regulators at the level of the membrane (Evans, 1974). Indeed, some of the effects of plant growth regulators on transport are very fast (e.g. ABA induced stomatal closure; Itai et al., 1978; and ABA inhibited ion uptake; Erlandsson et al., 1978) and may rely on direct interaction with the membrane. If plant growth regulators do have a direct effect on membranes, then it is possible that this may even be the principle mechanism of action (van Steveninck, 1976). Transport processes then may lead to changes in the nucleocytoplasmic environment and hence secondarily affect enzyme and gene activity (Göring and Mardanov, 1976).

Whether plant growth regulator actions generally are due primarily to membrane effects, which then indirectly affect the realisation of genetic information, still remains an unsolved and controversial problem, although there is no convincing reason why both actions should not occur independently or in parallel (Marmé, 1977), although this might require the action of multiple perceptors.

From the results obtained here, it appears unlikely that the mechanism of action of ABA in inducing turion formation or

inhibition of growth is to found at the level of the membrane. Clearly ABA induced stomatal closure (Jones and Mansfield, 1970) or inhibited stomatal opening (Horton, 1971), while certainly an example of one of the most studied and clear cut aspects of ABA control at the membrane level, has little relevance to ABA induced turion formation in the Lemnaceae, since these plants contain functionless stomata.

No rapid effect of ABA on ion transport was detected in S. polyrrhiza by the method of compartmental analysis. A small reduction in K^+ efflux across the plasmalemma with a concomitant increase in the cytoplasmic concentration of K^+ was observed after incubation of plantlets in ABA for 18 hours. However, the magnitude and rapidity of this change does not indicate the plasmalemma as the primary site of action of ABA, and interestingly bears no similarity to the effect of ABA on inhibiting K^+ uptake into stomatal guard cells (Mansfield and Jones, 1971; Horton and Moran, 1972) or in a variety of other tissues (Marrè, 1979).

While my work indicated that there are no obvious ion effects causal to turion formation or involved with turion initiation, the ionic relations of the fully developed turion are extremely different to those of the vegetative frond. The results tentatively suggest that the fully mature turion bears much in common with dormant storage tissue. The vastly increased permeability of the plasmalemma to Ca^{2+} in the turion indicates a loss of control of ion transport perhaps by a mechanism involving a decreased ability for a Ca^{2+} efflux pump to operate and/or a hyperpolarisation of the membrane potential (Hartung et al., 1980). In this instance further

speculation without detailed measurement of the membrane potential in fronds and turions of S. polyrrhiza would be an indulgence. The turion also appears to have lost the ability to maintain a strict control on K^+ and Cl^- uptake, in both cases resulting in an increased rate of efflux with a low cytoplasmic content.

The Ca^{2+} ionophore A23187 and the K^+ ionophore valinomycin, either alone or in conjunction with ABA proved neither facilitators or inhibitors of turion formation. This is consistent with the argument that whatever ionic changes have occurred during the formation of the turion, they are a secondary consequence of turion formation, either due to the regulation of enzymes important in membrane transport, or by affecting energy metabolism and the availability of energy for transport. The latter mechanism seems unlikely in view of the fact that ATP levels did not appear to be limiting during turion formation.

It is sometimes forgotten that although ABA may specifically affect ion transport, ABA is a relatively lipophilic molecule especially when in the undissociated form and the cell membrane would probably present no barrier to it (Weyers and Hillman, 1979). This does not mean of course that the membrane environment would not be disrupted by the penetration of ABA (Lea and Collins, 1979). It is quite possible that even dramatic effects at the cell membrane especially those that occur rapidly, might have no relevance at all in the mechanism of ABA action in turion formation (cf. the effect of GA_3 on synthetic membrane systems; Wood and Paleg, 1972).

In summary therefore this work has outlined some of the ultrastructural, biochemical and molecular events occurring during

turion formation, and it is hoped that this might form a basis for comparison for any future work on the development of the dormant state. I have summarised the major findings in figure 80. The main difference in the biochemical events occurring during the development of both types of frond is that during the development of the vegetative frond, both protein and RNA synthesis decline at approximately the same stage after the main period of cell expansion. During the development of the turion however, protein synthesis declines quite early on in the cell division phase and RNA synthesis only towards the end of the short expansion phase. It is interesting that turion formation is reversible up to 3 days when RNA synthesis has declined by 50%. This overall inhibition of RNA synthesis may represent the onset of the irreversible events leading to the dormant state.

7·6 THE MOLECULAR BASIS OF SENSITIVITY TO ABA

One of the most interesting aspects of ABA induced turion formation was not only the very narrow range of ABA concentrations which were effective, but the narrow window of sensitivity of the cell during its development to ABA. Only cells within the sensitivity window could both perceive the signal and act upon it. This response is not without precedence in growth regulator research. The work of Wright (1961, 1966) has shown that developing wheat coleoptiles pass through a sequential pattern of sensitivity to gibberellin, cytokinin and auxin as the cells mature. More recently Le Pabic (1976) found differential effects of benzyladenine on the pigment contents and plastid ultrastructure in S. polyrrhiza

Figure 80

Summary of the events occurring during the development of the vegetative frond (steps 1, 2, 4, 6, and 8) and the turion (steps 1, 3, 5 and 7) of S. polyrrhiza (N).

Vegetative frond:

step 1 - 2 main period of cell division

step 2 - 6 main period of cell expansion

step 6 - 8 main period of cell separation and differentiation of the mesophyll

Turion:

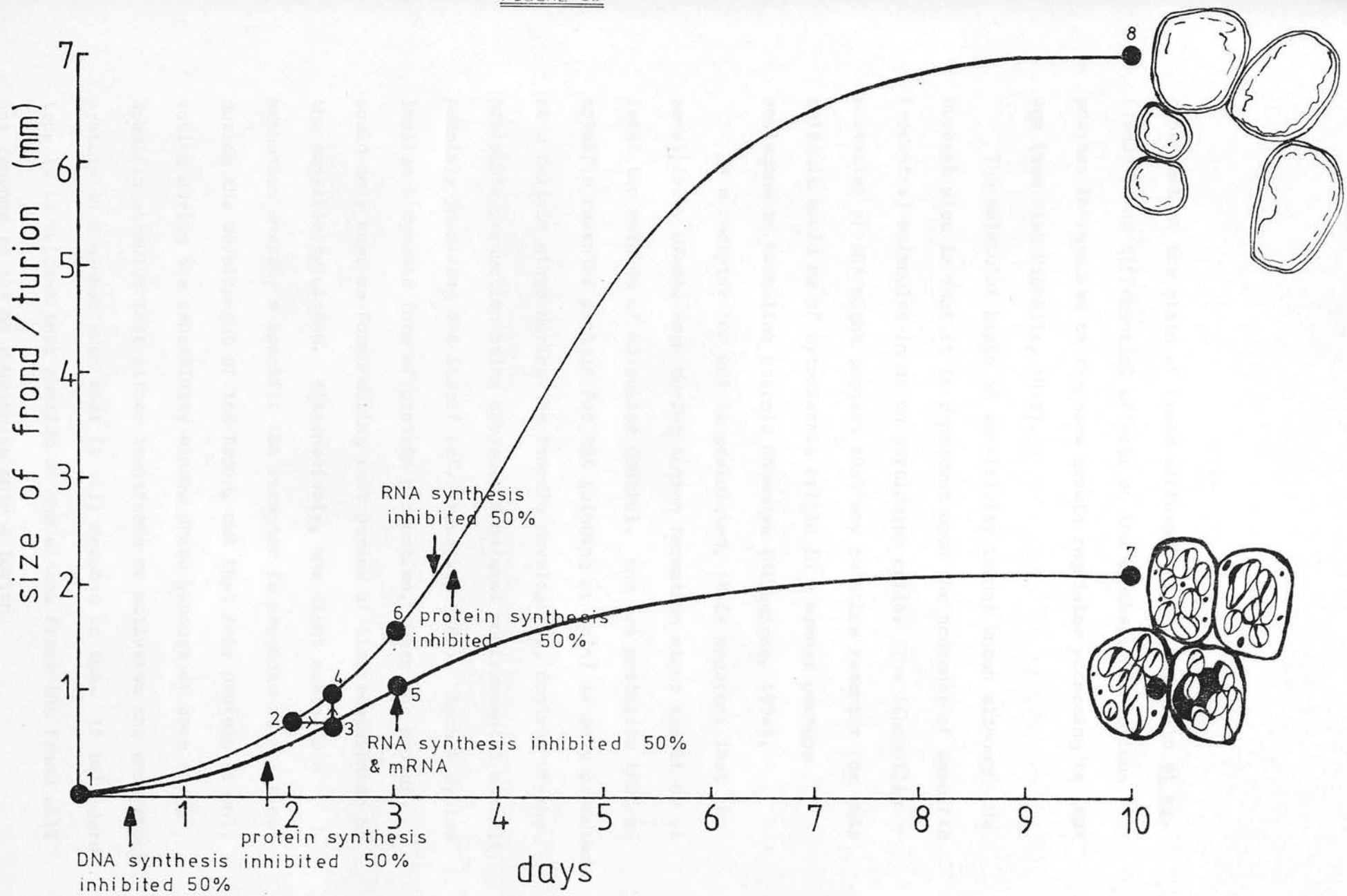
step 1 - 3 main period of cell division

step 3 - 5 main period of cell expansion

step 5 - 7 main period of starch and anthocyanin accumulation

Vegetative fronds below step 2 can switch to the turion developmental programme via step 3. Turions below step 3 can switch to the vegetative frond programme via step 4. After step 2 vegetative frond cells can no longer switch to the turion developmental programme and after step 5 turion cells can no longer switch to the vegetative frond programme.

FIGURE 80


size of frond / turion (mm)
7
6
5
4
3
2
1
days
1
2
3
4
5
6
7
8
9
10
DNA synthesis inhibited 50%
protein synthesis inhibited 50%
RNA synthesis inhibited 50% & mRNA
protein synthesis inhibited 50%
RNA synthesis inhibited 50%

dependent on the state of frond differentiation, and Naito et al. (1980) found differential effects on the synthesis of various enzymes in response to the same growth regulator according to leaf age (see also Nicholls, 1978).

The molecular basis of sensitivity is not known although the current view is that it is dependent upon the presence of specific (receptor) molecules in or on particular cells. The lipophilic character of ABA might suggest that any putative receptor for this molecule would be of cytoplasmic origin in a manner perhaps analagous to mammalian steroid hormones (Milborrow, 1974).

If a receptor for ABA is postulated, it is apparent that the sensitivity window seen during turion formation might result by at least two methods of molecular control. One can postulate that a specific receptor protein for ABA (binding protein) is only produced at a certain stage during the frond's development, control of the protein's production being subject to natural environmental stimuli, possibly involving ABA itself (cf. Trewavas, 1980). Such a system implies a dynamic form of protein production, where the protein would only ever be found during that period of time represented by the sensitivity window. Alternatively, one might envisage a mechanism whereby a specific ABA receptor is produced at all times during the development of the frond, but that this protein is only active during the sensitivity window phase because of some stage specific stimulus that either transforms or activates the endogenous protein in a manner such that it will respond to ABA. If this were true it is evident that outside of the window frame the frond will not respond to ABA by developing into a turion.

Since the Lemnacean frond appears always to respond to ABA with growth inhibition, with or without turion formation, at its simplest the inhibition response would seem to result from the tissue having a constant or tonic receptor presence (possibly by a mechanism involving negative co-operativity which would explain the wide concentration response range). One might extrapolate further and suggest that the turion forming phase of the ABA response might involve the presence of this tonic inhibitory receptor, and an additional component, integrally linked and dependent upon the first receptive inhibitory element that is produced or evident only subject to stimuli invoked by environmental changes.

The consequences of the above interpretation would be that turion formation would not occur in the absence of frond growth inhibition, but that the converse situation could occur. Thus we may obtain a mutant that could be inhibited by ABA but never capable of turion formation i.e. second receptor missing or inactive etc., while the converse would not be true. Therefore a mutant that is incapable of both responses to ABA requires only the absence of the growth inhibition receptor and consequently only a single genetic change would be necessary. If the receptor for both responses were independent, such a mutant could only arise by two separate genetic changes which although evidently possible, is not speculatively economical. The consequences of such an integral relationship between the two proteins may well be the type of mirror image curves observed by Stewart (1969).

One could of course postulate that a specific growth inhibition response may itself result in the expression of the second receptor

protein, which would than be the turion responsive component. In this way the turion forming response would be the second step in the tissue's response to ABA i.e. by the production of the receptor for turion formation or the inhibition of proteins which normally inhibit this receptor's production. Arguments based on the simultaneous presence of two proteins, one for inhibition, one for turion formation, differing only in their binding affinities for ABA might have to explain the apparent absence of a mutant of Spirodela polyrrhiza which forms turions in the absence of growth inhibition.

Plant growth regulator receptor research is still very much in its infancy, although several approaches to the problem have been made (Venis, 1977). It is felt that binding studies utilising S. polyrrhiza might be especially fruitful in the search for ABA receptors.

APPENDIX

The principle measuring methods employed in morphometry, generally known as stereology, allow information on volumes, surface areas, numbers of structures, and many other dimensions, to be derived from simple counting operations.

The basic operation in stereology consists of superimposing, on sectioned material, a suitable test system of points, lines or areas, according to the dimension sought. The theoretical principles of the various test systems employed have been discussed by Weibel (1963).

a) Volumetric analysis

Modern point counting techniques as described by Weibel and others, are a refinement of the principles developed by the geologist Delesse in 1846. Delesse found that the volume fraction Vv of a given component in a material can be estimated by measuring from a section, the area fraction Aa covered by a trans-section of the component (Weibel, 1973):

$$Vv = Aa \quad \ldots\ldots\ldots\

The volume contributions to the tissue of two components a and b are found from:

$$Vva = Ppa = \frac{Pa}{Pt} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots(2)$$

and

$$Vvb = Ppb = \frac{Pb}{Pt} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots(3)$$

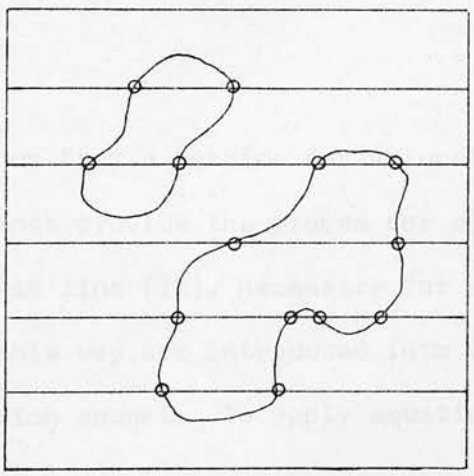
where Pt is the total number of points placed on the section, and Pa and Pb are the number of points lying on a and b respectively.

b) Surface area

The surface area of structures in a unit volume of tissue can be defined as their surface density Sv. In sections, a surface appears as a contour surrounding the sectioned component, the length of this contour on the unit area of section being proportional to Sv. Tomkeieff (1945) has shown that the number of intersections Ii of this surface with test lines of total length Lt is proportional to the surface density according to the formula:

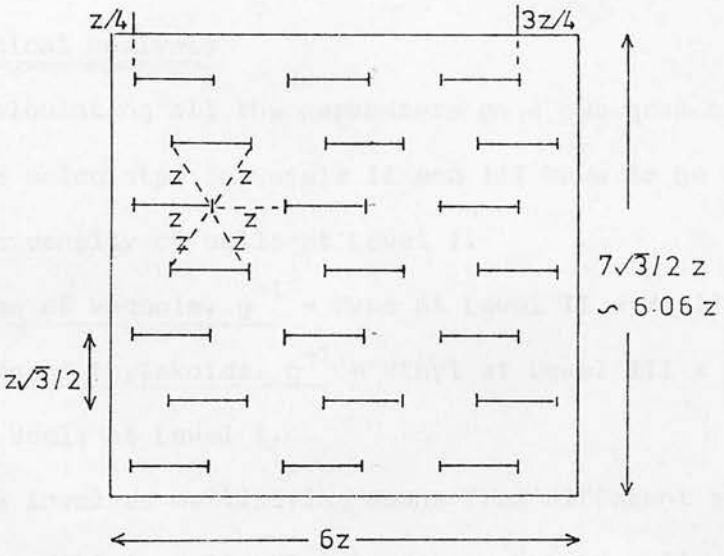
$$Sv = \frac{2.Ii}{Lt} = 2.Il \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots(4)$$

where Il is the number of intersections between the surface and the unit length of the test line (o).



c) Multipurpose test system

It has been shown that stereological measurements require various test systems: a lattice of test points for volumetric work, and test lines for surface measurements. A test system combining these requirements would thus allow both these parameters to be obtained from a section at one time. To this end a multipurpose test system has been developed (Weibel et al., 1966), which consists of a square frame enclosing 21 lines of equal length z. These are arranged in equidistant parallel rows, the distance between the end points being z in every direction.



The 42 end points thus form a lattice for volumetric estimation Pp, while the 21 test lines provide the probes for counting surface intersections per unit line (Il), necessary for surface estimations. Counts obtained in this way are introduced into equations (2) - (4) to derive the dimension sought. To apply equation (4) we take

Lt = 21z, and record Ii as the number of intersections per field, or

$$Sv = 2.Il = \frac{4.Ii}{Pt.z} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots(5)$$

Since the test system is coherent, it is possible to estimate Sv in the cytoplasm for example, by introducing cytoplasmic points Pc, instead of Pt:

$$Sv = \frac{4.Ii}{Pc.z} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots(6)$$

The basic unit of this test system allows a number of coherent test systems to be designed from the original pattern, according to one's needs.

d) Statistical analysis

In calculating all the parameters on a per gram basis, those parameters calculated in Levels II and III have to be multiplied by the volume density of cells at Level I.

e.g. Volume of vacuole. g^{-1} = Vvac at Level II x Vcell at Level I

and Volume of thylakoids. g^{-1} = Vthyl at Level III x Vcyt at Level II x Vcell at Level I.

Since this involves multiplying means from different sampling levels, the new mean obtained is not a true mean but an estimate. A true mean can only be obtained if the samples are totally independent of one another, which is probably not the case in this instance e.g. cell density in the tissue is probably positively correlated to the cytoplasm density in the cell, since the development of air spaces and vacuoles both increase temporally. The parameters were however calculated in this way, simply to aid comparison between the samples at one comprehensive level.

BIBLIOGRAPHY

Al-Shalan, I. and R. Kandeler (1978). Biochem. Physiol. Pflanzen. 172, 521-529.

Altman, A and R. Goren (1974). Physiol. Plant. 30, 240-245.

Alvim, R., E.W. Hewitt and P.F. Saunders (1976). Plant Physiol. 57, 474-476.

Alvim, R., P.F. Saunders and R.S. Barros (1979). Plant Physiol. 63, 774-777.

Anderson, J.L., W.W. Thompson and J.A. Swader (1973). Can. J. Bot. 51, 1619-1622.

Andres, J. and H. Smith (1976). Plant Sci. Lett. 6, 315-318.

Arber, A. (1919). Proc. Roy. Soc. B. 91, 96-103.

Arber, A. (1920). Water plants. A study of aquatic angiosperms. Cambridge University Press.

Arnon, D.I. (1949). Plant Physiol. 24, 1-15.

Ashby, E. and E. Wangermann (1949). Nature 164, 187.

Atkin, R.K. and B.I.S. Srivastava (1970). Physiol. Plant. 23, 304-315.

Bauer, R., W. Huber and N. Sankhla (1976). Z. Pflanzenphysiol. 77, 237-246.

Behl, R. and W.D. Jeschke (1979). Z. Pflanzenphysiol. 95, 335-353.

Berrie, A.M.M., D. Buller, R. Don and W. Parker (1979). Plant Physiol. 63, 758-764.

Best, E.P.H. (1979). Physiol. Plant. 45, 399-406.

Bex, J.H.M. (1972a). Planta 103, 1-10.

Bex, J.H.M. (1972b). Planta 103, 11-17.

Bex, J.H.M. (1972c). Acta Bot. Neerl. 21, 203-210.

Bird, I.F., M.J. Cornelius, A.J.Keys and C.P. Whittingham (1974). Phytochemistry 13, 59-64.

Blenkinsop, P.G. (1973). Ph.D. Thesis. Edinburgh University.

Bonner, W.M. and R.A. Laskey (1974). Eur. J. Biochem. 46, 83-88.

Bradford, M.M. (1976). Anal. Biochem. 72, 248-254.

Brady, C.J., N.S. Scott and R. Munns (1974). R. Soc. N. Z. Bull. 12, 403-409.

Brooks, J.S. (1940). Ph.D. Thesis. Cornell University.

Brunaud, A. (1974a). C. R. Acad. Sci. Paris Ser. D. 278, 1183-1186.

Brunaud, A. (1974b). C. R. Acad. Sci. Paris Ser. D. 278, 2019-2022.

Bryant, J.A., D.C. Wildon and D. Wong (1974). Planta 118, 17-24.

Burton, K. (1956). Biochem. J. 62, 315-323.

Camm, E.L. and G.H.N. Towers (1973). Phytochemistry 12, 961-973.

Chen, D. and D.J. Osborne (1970). Nature 266, 1157-1160.

Chen, S.S.C. and W.M. Park (1976). Taiwania 21, 50-51.

Chiu, M.M. and R.H. Falk (1975). Cytologia 40, 313-322.

Chrispeels, M.J. and J.E. Varner (1966). Nature 212, 1066-1067.

Chrispeels, M.J. and J.E. Varner (1967). Plant Physiol. 42, 1008-1016.

Clark, N.A. (1930). Science 71, 268-269.

Claus, W.D. (1972). New Phytol. 71, 1081-1095.

Cline, M.G. and M.M. Rehm (1974). Int. Conf. Plant Growth Subst., 8th, Tokyo 582-592.

Cocucci, M.C. and E. Marrè (1973). Plant Sci. Lett. 1, 293-301.

Collins, P. and H. Diehl (1960). Anal. Chim. Acta 22, 125-127.

Constabel, F., J.P. Shyluk and O.L. Gamborg (1971). Planta 96, 306-316.

Corgan, J.N. and G.C. Martin (1971). Hort. Sci. 6, 405-406.

Cornforth, J.W., B.V. Milborrow, G. Ryback and P.F. Wareing (1965). Nature 205, 1269-1270.

Covey, S.N. (1972). B.Sc. Thesis. University of Nottingham.

Craker, L.E. (1975). Phytochemistry 14, 151-153.

Craker, L.E, L.A. Standley and M.J. Starbuck (1971). Plant Physiol. 48, 349-352.

Cram, W.J. and M.G. Pitman (1972). Aust. J. Biol. Sci. 25, 1125-1132.

Cuatrecasas, P. (1974). Annu. Rev. Biochem. 43, 169-214.

Cummins, W.R., H. Kende and K. Raschke (1971). Planta 99, 347-351.

Czopek, M. (1959). Acta Biol. Cracov. Ser. Bot. 2, 76-90.

Czopek, M. (1962). Acta Soc. Bot. Pol. 31, 715-722.

Czopek, M. (1963). Acta Soc. Bot. Pol. 32, 199-211.

Czopek, M. (1964). Bull. Acad. Pol. Sci. II. 12, 177-182.

Czopek, M. (1967). Acta Soc. Bot. Pol. 36, 87-96.

Daubs, E.H. (1965). Illinois Biol. Monog. 34. Univ. Illinois Press, Urbana.

Davies, D.D. and T.J. Humphrey (1978). Plant Physiol. 61, 54-58.

DeKock, P.C., P.W. Dyson, A. Hall and F. Grabowska (1975). Potato Res. 18, 573-581.

DeKock, P.C., D. Vaughan and A. Hall (1978). New Phytol. 81, 505-511.

Dennis, F.G., G.C. Martin, P. Gaskin and J. MacMillan (1978). J. Am. Soc. Hortic. Sci. 103, 314-317.

Dittrich, P. and K. Raschke (1977). Planta 134, 77-81.

Dunlop, J. (1973). Planta 112, 159-167.

During, H. and O. Bachmann (1975). Physiol. Plant. 34, 201-203.

Duysen, M.E. and T.P. Freeman (1976). Amer. J. Bot. 63, 1134-1139.

Eagles, C.F. and P.F. Wareing (1964). Physiol. Plant. 17, 697-709.

El-Antably, H.M.M., P.F. Wareing and J. Hillman (1967). Planta 73, 74-90.

Eliasson, L. and E. Tillberg (1976). Int. Conf. Plant Growth Subst., 8th, Lausanne, Switz. 36, 84-85.

Elliott, D.C. (1977). Aust. J. Plant Physiol. 4, 39-50.

Ellis, J.R. (1979). Trends in Biochemical Science 4, 241-244.

Ellis, J.R., P.E. Highfield and J. Silverthorne (1977). Proc. Int. Congr. Photosynthesis 4th, 497-506.

Emmerson, J.G. and L.E. Powell (1978). J. Am. Soc. Hortic. Sci. 103, 677-680.

Erlandsson, G., S. Pettersson and S-B. Svensson (1978). Physiol. Plant. 43, 380-384.

Esau, K. (1965). Plant Anatomy, 2nd edition. J. Wiley and Sons, Inc., New York.

Evans, M.L. (1974). Annu. Rev. Plant Physiol. 25, 195-223.

Fagerlind, F. and A. Massalski (1974). Sven. Bot. Tidskr. 68, 64-93.

Fraser, G.G. (1974). Mauri Ora 2, 147-156.

Furuya, M. and K.V. Thimann (1964). Arch. Biochem. Biophys. 108, 109-116.

Gayler, K.R. and K.T. Glasziou (1969). Planta 84, 185-194.

Giles, K.L., M.F. Beardsell and D. Cohen (1974). Plant Physiol. 54, 208-212.

Glinka, Z. (1973). Plant Physiol. 51, 217-219.

Glinka, Z. and L. Reinhold (1971). Plant Physiol. 48, 103-105.

Glinka, Z. and L. Reinhold (1972). Plant Physiol. 49, 602-606.

Godziemba-Czyz, J. (1969a). Acta Soc. Bot. Pol. 38, 437-452.

Godziemba-Czyz, J. (1969b). Acta Soc. Bot. Pol. 39, 421-443.

Goldthwaite, J. (1974). Plant Physiol. 54, 399-403.

Gordon, W.R. and W.L. Koukkari (1978). Plant Physiol. 62, 612-615.

Göring, H. and A.A. Mardanov (1976). Biol. Rdsch. 14, 177-189.

Gregor, H.D. (1974). Protoplasma 80, 273-277.

Guern, J. (1965). Annales des Sciences Naturelles, Botanique, Paris, 12^{e} Serie. 6, 1-156.

Guinn, G. (1966). Plant Physiol. 41, 689-695.

Gunning, B.E.S. and M.W. Steer (1975). Plant Cell Biology, an ultrastructural approach. Edward Arnold.

Guppy, H.B. (1895). J. Linn. Soc. Bot. 30, 323-330.

Guruprasad, K.N. and M.M. Laloraya (1980). Plant Sci. Lett. 19, 73-79.

Haber, A.H., D.E. Foard and S.W. Perdue (1969). Plant Physiol. 44, 463-467.

Haberlandt, G. (1914). Physiological Plant Anatomy. MacMillan and Co. Ltd., London.

Haddon, L. and D.H. Northcote (1976). J. Cell. Sci. 20, 47-55.

Hanson, W.C. (1950). J. Sci. Fo. Agric. 1, 172-173.

Harrison, M.A. and P.F. Saunders (1975). Planta 123, 291-298.

Hartung, W., C.I. Ullrich-Eberius, U. Lüttge, M. Bocher and A. Novacky (1980). Planta 148, 256-261.

Heber, U. (1974). Annu. Rev. Plant Physiol. 25, 393-421.

Hedley, C.L. and J.L. Stoddart (1972). J. Exp. Bot. 23, 490-501.

Hegelmaier, F. (1868). Die Lemnaceen-eine monographische Untersuchung. Wilhelm Engelmann, Leipzig.

Hegelmaier, F. (1895). Bot. Jahrb. 21, 268-305.

Heldt, H.W. (1976). In Encyclopedia of Plant Physiology, Transport in Plants, Vol. 3 (ed. C.R. Stocking and U. Heber), pp. 137-143, Springer Verlag, Berlin and New York.

Hemberg, T. (1949a). Physiol. Plant. 2, 24-36.

Hemberg, T. (1949b). Physiol. Plant. 2, 37-44.

Hemberg, T. (1978). Physiol. Plant. 43, 65-67.

Henry, E.W. (1975). J. Microscopie Biol. Cell. 22, 109-116.

Henssen, A. (1954). Flora 141, 523-566.

Hicks, L.E. (1937). Amer. Mid. Nat. 18, 774-789.

Higgins, T.J.V., J.A. Zwar and J.V. Jacobsen (1976). Nature 260, 166-169.

Higgins, T.J.V., J.A. Zwar and J.V. Jacobsen (1977). In Nucleic acids and protein synthesis in plants (ed. J.H. Weil and L. Bogorad), 261, pp. 481-486, C.N.R.S., Paris.

Hillman, W.S. (1961). Bot. Rev. 27, 221-287.

Hillman, W.S. (1975). Amer. J. Bot. 62, 537-540.

Hillman, W.S. (1976). Science 193, 453-458.

Hillman, W.S. (1977). Nature 266, 833-835.

Hiron, R.W.P. (1974). Ph.D. Thesis. University of London.

Ho, D.T.-H. (1979). Plant Physiol. 63, (Suppl.) 79.

Ho, D.T.-H. and J.E. Varner (1974). Proc. Natl. Acad. Sci. USA 71, 4783-4786.

Ho, D.T.-H. and J.E. Varner (1976). Plant Physiol. 57, 175-178.

Hocking, T.J. and J.R. Hillman (1975). Planta 125, 235-242.

Hoffman, F. and U. Kull (1974). Experentia 30, 746-747.

Hoffman, J.F. (1840). Weigmann's Arch. F. Naturgeschich., Berlin, Jahrg. 6, 138-163.

Horen, F. van, (1870). J. Bot. 8, 36-40.

Horton, R.F. (1971). Can. J. Bot. 49, 583-585.

Horton, R.F. and K.R. Bruce (1972). Can. J. Bot. 50, 1915-1917.

Horton, R.F. and L. Moran (1972). Z. Pflanzenphysiol. 66, 193-196.

Huber, W., F. Kreutmeier and N. Sankhla (1977). Z. Pflanzenphysiol. 81, 234-247.

Humphreys, T. (1973). In Molecular techniques and approaches in developmental biology (ed. M.J. Chrispeels), pp. 141-163, John Wiley, New York.

Ihle, J.N. and L. Dure (1970). Biochem. Biophys. Res. Commun. 38, 995-1001.

Itai, C., J.D.B. Weyers, J.R. Hillman, H. Meidner and C. Willmer (1978). Nature 271, 652-653.

Jacobs, D.L. (1947). Ecol. Monog. 17, 437-469.

Jacobsen, J.V. and T.J.V. Higgins (1978a). In Plant Growth Substances and Related Compounds. A Comprehensive Treatise (ed. D.S. Letham, P.B. Goodwin and T.J. Higgins), vol. I, pp. 515-582. Elsevier, North Holland Biomedical Press, Amsterdam.

Jacobsen, J.V. and T.J.V. Higgins (1978b). In Plant Growth Substances and Related Compounds. A Comprehensive Treatise (ed. D.S. Letham, P.B. Goodwin and T.J. Higgins), vol. I, pp. 583-621. Elsevier, North Holland Biomedical Press, Amsterdam.

Jacobsen, J.V., T.J.V. Higgins and J.A. Zwar (1980). In The Plant seed: Development, Preservation and Germination (ed. I.

Rubenstein), pp. 241-258. Academic Press, New York.

Jaffe, L.F. (1969). Dev. Biol. Supp. 3, 83-111.

Jarvis, B.C., B. Frankland and J.H. Cherry (1968). Plant Physiol. 43, 1734-1736.

Jensen, R.G. (1980). In The Biochemistry of Plants. A comprehensive treatise, Vol. I, The Plant Cell (ed. N.E. Tolbert), pp. 273-313. Academic Press, London.

Jones, M.G.K. (1979). Plant Cell. Environment 2, 227-234.

Jones, M.G.K., W.H. Outlaw and O.H. Lowry (1977). Plant Physiol. 60, 379-383.

Jones, R.J. and T.A. Mansfield (1970). J. Exp. Bot. 21, 714-719.

Jones, R.L. and J.V. Jacobsen (1978). Bot. Mag. Tokyo Spec. Issue 1, 83-99.

Kandeler, R. (1955). Zeit. Bot. 43, 61-71.

Kang, B.G. and S.P. Burg (1973). Planta 110, 227-235.

Karmoker, J.L. and R.F.M. van Steveninck (1979). Physiol. Plant. 45, 453-459.

Kasamo, K. (1979). Plant. Cell Physiol. 20, 293-300.

Kasinov, V.B. and G.V. Kasinova (1974). Int. J. Chronobiol. 2, 47-52.

Khan, A.A. (1972). Int. Conf. Plant Growth Subst., 7th, 207-215.

Khan, A.A. and C.E. Heit (1969). Biochem. J. 113, 707-712.

Kirk, C.A. van, and K. Raschke (1978). Plant Physiol. 61, 474-475.

Klein, A.O. and C.W. Hagen (1961). Plant Physiol. 36, 1-9.

Knypl, J.S. (1977). Biochem. Physiol. Pflanzen. 171, 289-298.

Knypl, J.S. (1978). Z. Pflanzenphysiol. 90, 265-277.

Kondo, T. and T. Tsudzuki (1978). Plant. Cell Physiol. 19, 1465-1473.

Kopperschläger, G., W. Diezel, B. Bierwagen and E. Hofmann (1969). FEBS Lett. 5, 221-224.

Koshimizu, K., M. Inui, H. Fukui and T. Mitsui (1968). Agric. Biol. Chem. 32, 789-791.

Krause, J. and D. Strack (1979). Z. Pflanzenphysiol. 95, 183-187.

Kriedemann, P.E., B.R. Loveys, G.L. Fuller and A.C. Leopold (1972). Plant Physiol. 49, 842-847.

Kuraishi, S. (1973). Plant. Cell Physiol. 14, 689-718.

Lacor, M.A.M. (1969). Acta Bot. Neerl. 18, 550-557.

Lado, P., F. Rasi-Caldogno and R. Colombo (1977). Plant Sci. Lett. 9, 93-101.

Laemmli, U.K. (1970). Nature 227, 680-685.

Landolt, E. (1957). Ber. Schweiz. Bot. Ges. 67, 271-410.

Landolt, E. (1975). Aquatic Botany 1, 345-363.

Lautner, V. and Z. Müller (1954). Sbornik Ceskoslov. Akad. Zemedel. Ved. A. 27, 333-354.

Lawalrée, A. (1943). La Cellule 49, 335-382.

Lawalrée, A. (1945). Bull. Soc. Roy. Bot. Belg. 77, 27-38.

Lea, E.J.A. and J.C. Collins (1979). New Phytol. 82, 11-18.

Leaver, C.J. (1973). Biochem. J. 135, 237-240.

Leaver, C.J. and J. Ingle (1971). Biochem. J. 123, 235-243.

Ledbetter, M.C. and K.R. Porter (1970). Introduction to the fine structure of plant cells. Springer Verlag, Berlin.

Lenton, J.R., V.M. Perry and P.F. Saunders (1972). Planta 106, 13-22.

Le Pabic, C. (1972). C.R. Acad. Sci. Paris Ser. D. 275, 2339-2342.

Le Pabic, C. (1976). J. Microscopie Biol. Cell. 25, 181-186.

Lilley, R. McC., C.J. Chon, A. Mosbach and H.W. Helldt (1977). Biochim. Biophys. Acta 460, 259-272.

Lin, W. and J.B. Hanson (1974). Plant Physiol. 54, 250-256.

Lindeman, W. (1973). Acta Bot. Neerl. 22, 553-568.

Loening, U.E. (1967). Biochem. J. 102, 251-257.

Loppert, H. (1979). Planta 144, 311-315.

Loppert, H., W. Kronberger and R. Kandeler (1978). Planta 138, 133-136.

Loveys, B.R. and P.E. Kriedemann (1973). Physiol. Plant. 28, 476-479.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). J. Biol. Chem. 193, 265-270.

Lubin, M. (1964). In The cellular functions of membrane transport (ed. J.F. Hoffman), pp. 193-211. Prentice-Hall, Englewood Cliffs, New Jersey.

Macklon, A.E.S. (1975a). Planta 122, 109-130.

Macklon, A.E.S. (1975b). Planta 122, 131-141.

Macklon, A.E.S. (1976). J. Exp. Bot. 27, 651-657.

Macklon, A.E.S. and N. Higinbotham (1970). Plant Physiol. 45, 133-138.

MacRobbie, E.A.C. (1977). Int. Rev. Biochem. 13, 211-247.

Magar, M.E. (1972). Data Analysis in Biochemistry and Biophysics. Academic Press, New York.

Maheshwari, S.C. (1954). Phytomorphology 4, 355-365.

Maheshwari, S.C. and R.N. Kapil (1963). Amer. J. Bot. 50, 677-686.

Maillard-Sevhonkian, S. and P.-E. Pilet (1978). Plant. Cell Physiol. 19, 811-817.

Maksymowych, R. (1973). Analysis of leaf development. Cambridge University Prees, London.

Malek, L. and Y. Oda (1979). Plant Physiol. Supp. 63, 156.

Mallery, C.H. (1979). Plant. Cell Physiol. 20, 689-696.

Mans, R.V. and A.D. Novelli (1961). Arch. Biochem. Biophys. 94, 48-53.

Mansfield, T.A. and R.J. Jones (1971). Planta 101, 147-158.

Marmé, D. (1977). Annu. Rev. Plant Physiol. 28, 173-198.

Marrè, E. (1979). Annu. Rev. Plant Physiol. 30, 273-288.

Mason, H.L. (1938). Madroño 4, 241-251.

McCombs, P.J.A. and R.K. Ralph (1972). Biochem. J. 129, 403-417.

McLaren, J.S. (1976). Ph.D. Thesis. University of Nottingham.

McLaren, J.S. and H. Smith (1976). New Phytol. 76, 11-20.

McLaren, J.S. and H. Smith (1977). Phytochemistry 16, 219-221.

McLay, C.L. (1976). Freshwater Biol. 6, 125-136.

McWha, J.A. and D. Jackson (1976). J. Exp. Bot. 27, 1004-1008.

Meidner, H. and T.A. Mansfield (1968). Physiology of stomata. McGraw-Hill, London.

Melaragno, J.E. (1974). Ph.D. Thesis. University of Pittsburgh.

Melaragno, J.E. and M.A. Walsh (1976). Amer. J. Bot. 63, 1145-1157.

Milborrow, B.V. (1974). Annu. Rev. Plant Physiol. 25, 259-307.

Milborrow, B.V. (1978). In Phytohormones and related compounds. A comprehensive treatise, Vol. I, (ed. D.S. Letham, P.B. Goodwin and T.J. Higgins), pp. 295-347. Elsevier, North Holland Press, Amsterdam.

Milborrow, B.V. and D.R. Robinson (1973). J. Exp. Bot. 24, 537-548.

Mittelheuser, C. and R.F.M. van Steveninck (1969). Nature 221, 281-282.

Mittleheuser, C.J. and R.F.M. van Steveninck (1971). Planta 97, 83-86.

Mondal, M.H., W.A. Brun and M.L. Brenner (1978). Plant Physiol. 61, 394-397.

Moreland, D.E., G.G. Hussey, C.R. Shriner and F.S. Farmer (1974). Plant Physiol. 54, 560-563.

Morris, P.F. and W.G. Barker (1977). Can. J. Bot. 55, 1926-1932.

Mozer, T.J. (1980). Cell 20, 479-485.

Naito, K., T. Ebato, Y. Endo and S. Shimizu (1980). Z. Pflanzenphysiol. 96, 95-102.

Natr, L., B.T. Watson and P.E. Weatherley (1974). Ann. Bot. 38, 589-593.

Neales, T.F. and L.D. Incoll (1968). Bot. Rev. 34, 107-125.

Neuhof, V. (1973). Micromethods in molecular biology. Chapman and Hall, London.

Newton, R.J. (1974). Physiol. Plant. 30, 108-112.

Newton, R.J. (1977). Amer. J. Bot. 64, 45-49.

Newton, R.J., D.R. Shelton, S. Disharoon and J.E. Duffey (1978). Amer. J. Bot. 65, 421-428.

Obendorf, R.L. and A. Marcus (1974). Plant Physiol. 53, 779-781.

O'Farrell, P.H. (1975). J. Biol. Chem. 250, 4007-4021.

Osborne, D.J. (1967). Symp. Soc. Exp. Biol. 21, 305-322.

Overbeek, J. van, and M.I.R. Mason (1968). Acta Bot. Neerl. 17, 441-444.

Overbeek, J. van, J.E. Loeffler and M.I.R. Mason (1967). Science 156, 1497-1499.

Palmiter, R.D. (1974). Biochemistry 13, 3606-3615.

Pan, S.M. and S.S.C. Chen (1976). Taiwania 21, 248-250.

Paranjothy, K. and P.F. Wareing (1971). Planta 99, 112-119.

Pearson, J.A. and P.F. Wareing (1969). Nature 221, 672-673.

Pelc, S.R. (1968). Nature 219, 162-163.

Perry, T.O. (1968). Plant Physiol. 43, 1866-1869.

Perry, T.O. and O.R. Byrne (1969). Plant Physiol. 44, 784-785.

Perry, T.O. and H. Hellmers (1973). Bot. Gaz. 134, 283-289.

Phillips, R. and G.G. Henshaw (1977). J. Exp. Bot. 28, 785-794.

Phillips, I.D.J. and P.F. Wareing (1959). J. Exp. Bot. 10, 504-514.

Pierce, W.S. and N. Higinbotham (1970). Plant Physiol. 46, 666-673.

Pieterse, A.H., P.R. Bhalla and P.S. Sabharwal (1970). Acta Bot. Neerl. 19, 901-905.

Pilet, P.E. (1970). J. Exp. Bot. 21, 446-451.

Pitman, M.G. (1963). Aust. J. Biol. Sci. 16, 647-668.

Pirson, A. and E. Göllner (1953). Flora 140, 485-498.

Poskuta, J., R. Antoszewski and M. Faltynowicz (1972). Photosynthetica 6, 370-374.

Poulson, R. and L. Beevers (1970). Plant Physiol. 46, 782-785.

Powell, L.E. (1976). Hort. Sci. 11, 498-499.

Radin, J.W. and R.N. Trelease (1976). Plant Physiol. 57, 902-905.

Raghavendra, A.S., I.M. Rao and V.S.R. Das (1976). Plant Sci. Lett. 6, 111-115.

Ralph, R.K. and A.R. Bellamy (1964). Biochim. Biophys. Acta 87, 9-16.

Raschke, K. (1975). Annu. Rev. Plant Physiol. 26, 309-340.

Reed, N.R. and B.A. Bonner (1974). Planta 116, 173-185.

Reynolds, E.S. (1963). J. Cell. Biol. 17, 209-212.

Reznik, H. and R. Menschick (1969). Z. Pflanzenphysiol. 61, 348-349.

Rimon, D. (1964). Isr. J. Bot. 13, 24-29.

Rimon, D. and E. Galun (1967). Plant. Cell Physiol. 8, 283-291.

Rimon, D. and E. Galun (1968). Bot. Gaz. 129, 138-144.

Roberts, B.E. and B.M. Paterson (1973). Proc. Natl. Acad. Sci. USA 70, 2330-2334.

Robinson, P.M. and P.F. Wareing (1964). Physiol. Plant. 17, 314-323.

Roe, J.H. (1934). J. Biol. Chem. 107, 15-22.

Rombach, J. (1978). Photochem. Photobiol. 27, 781-786.

Rombach, J. and C.J.P. Spruit (1968). Acta Bot. Neerl. 17, 445-454.

Ryć, M. and St. Lewak (1980). Z. Pflanzenphysiol. 96, 195-202.

Saeger, A. (1930). Bull. Torrey. Bot. Club. 57, 117-122.

Saks, Y., M. Negbi and I. Ilan (1980). Aust. J. Plant Physiol. 7, 73-79.

Sankhla, N. and W. Huber (1974). Physiol. Plant. 30, 291-294.

Sankhla, N. and W. Huber (1975). Z. Pflanzenphysiol. 74, 237-271.

Sankhla, N. and W. Huber (1979). Z. Pflanzenphysiol. 91, 7-15.

Sarrouy-Balat, H., M. Delseny and R. Julien (1973). Plant Sci. Lett. 1, 287-292.

Saunders, P.F. and R.H. Poulson (1968). In Biochemistry and Physiology of Plant Growth Substances (ed. F. Wightman and G. Setterfield), pp. 1581-1591. Runge Press, Ottawa.

Saunders, P.F., M.A. Harrison and R. Alvim (1974). In Plant Growth Substances 1973, pp. 871-881. Tokyo, Hirokawa.

Scharfelter, E., T. Rattenburg and R. Kandeler (1978). Z. Planzenphysiol. 87, 445-454.

Schmidt, G. and S.J. Thannhauser (1945). J. Biol. Chem. 161, 83-89.

Sculthorpe, C.D. (1967). Biology of aquatic vascular plants. St. Martin Press.

Servattaz, O., D. Castelli and C.P. Longo (1975). Plant Sci. Lett. 4, 361-368.

Shaner, D.L., S.M. Mertz and C.J. Arntzen (1975). Planta 122, 79-90.

Sheppard, C.W. (1962). Basic principles of the tracer method. Wiley and Sons, New York.

Shone, M.G.T. (1968). J. Exp. Bot. 19, 468-485.

Sibaski, T. and Y. Oda (1979). Plant. Cell Physiol. 20, 563-571.

Sloover, J-L. de, (1966). Natur. Belg. 47, 443-456.

Smith, H. (1975). Phytochrome and Photomorphogenesis. McGraw-Hill, New York.

Smith, H. and T.H. Attridge (1970). Phytochemistry 9, 487-495.

Smith, R.H., A.M. Schubert and C.R. Benedict (1974). Plant Physiol. 54, 197-200.

Spanswick, R.M. and E.J. Williams (1965). J. Exp. Bot. 16, 463-473.

Spector, T. (1978). Anal. Biochem. 86, 142-146.

Spencer, P.W. and J.S. Titus (1972). Plant Physiol. 49, 746-750.

Srivastava, B.I.S. (1968). Biochim. Biophys. Acta 169, 534-536.

Staden, J. van, and C.H. Bornman (1969). Planta 85, 157-159.

Staden, J. van, and C.H. Bornman (1970a). J. S. Afr. Bot. 36, 9-12.

Staden, J. van, and C.H. Bornman (1970b). J. S. Afr. Bot. 36, 207-214.

Steinhardt, R., R. Zucker and G. Schatten (1977). Dev. Biol. 58, 185-196.

Steup, M., D.G. Peavey and M. Gibbs (1976). Biochem. Biophys. Res. Commun. 72, 1554-1561.

Steveninck, R.F.M. van, (1972). Z. Pflanzenphysiol. 67, 282-286.

Steveninck, R.F.M. van, (1975). Annu. Rev. Plant Physiol. 26, 237-258.

Steveninck, R.F.M. van, (1976). In Encyclopedia of Plant Physiology, Transport in Plants, Vol 2B (ed. C.R. Stocking and U. Heber), pp. 307-342. Springer Verlag, Berlin and New York.

Stewart, G.R. (1969). Nature 221, 61-62.

Stewart, G.R. and D. Rhodes (1977). New Phytol. 79, 257-268.

Stewart, G.R. and H. Smith (1972). J. Exp. Bot. 23, 875-885.

Switzer, R.C., C.R. Merrill and S. Shifrin (1979). Anal. Biochem. 98, 231-237.

Taiz, L. (1975). Plant Physiol. Supp. 56, 45.

Tanaka, O. and A. Takimoto (1977). Plant. Cell Physiol. 18, 27-34.

Tasseron-de Jong, J.G. and H. Veldstra (1971). Physiol. Plant. 24, 235-238.

Thimann, K.V. and Y.H. Edmondson (1949). Arch. Biochem. 22, 33-53.

Thimann, K.V., Y.H. Edmondson and B.S. Radner (1951). Arch. Biochem. 34, 305-323.

Thompson, C.H. (1898). Mo. Bot. Gard. Rep. 9, 21-42.

Tillberg, E., M. Holmvall and T. Eriesson (1979). Physiol. Plant. 46, 5-12.

Tobin, E.M. (1978). Proc. Natl. Acad. Sci. USA 75, 4749-4753.

Tobin, E.M. and J.L. Suttie (1980). Plant Physiol. 65, 641-647.

Tomkeieff, S.I. (1945). Nature 155, 24.

Trewavas, A. (1970). Plant Physiol. 45, 742-751.

Trewavas, A. (1972a). Plant Physiol. 49, 40-46.

Trewavas, A. (1972b). Plant Physiol. 49, 47-51.

Trewavas, A. (1973). Plant Physiol. 51, 760-767.

Trewavas, A. (1980). Phytochemistry 19, 1303-1308.

Trewavas, A. (1981). Plant, Cell. Enviromment 4, 203-228.

Tuan, D.Y.H. and J. Bonner (1964). Plant Physiol. 39, 768-772.

Tucker, D.J. and T.A. Mansfield (1971). Planta 98, 157-163.

Ullrich-Eberius, C.I., A. Novacky and U. Lüttge (1978). Planta 139, 149-153.

Vegis, A. (1964). Annu. Rev. Plant Physiol. 15, 185-224.

Venis, M.A. (1977). Adv. Bot. Res. 5, 53-88.

Villiers, T.A. (1968). Planta 82, 342-354.

Vince, D. (1968). Planta 82, 261-279.

Vintejoux, C. (1969). C.R. Acad. Sci. Paris Ser. D. 269, 44-47.

Wagner, T. (1973). Funktionieren die Stomata bei Schwimmblattern? Staatsexamensarbut. Bot. Inst. d. TU. München.

Walbot, V., M. Clutter and I. Sussex (1975). Plant Physiol. 56, 570-574.

Walker, N.A. and M.G. Pitman (1976). In Encyclopedia of Plant Physiology, Transport in Plants, Vol. 2A, Cells (ed. U. Luttge and M.G. Pitman), pp. 93-126. Springer Verlag, Berlin.

Walton, D.C. and E. Sondheimer(1968). Plant Physiol. 43, 467-469.

Walton, D.C., G. Soofi and E. Sondheimer (1970). Plant Physiol. 45, 37-40.

Wareing, P.F. (1954). Physiol. Plant. 7, 261-277.

Wareing, P.F. (1956). Annu. Rev. Plant Physiol. 7, 191-214.

Wareing, P.F. and I.D.J. Phillips (1970). The control of growth and differentiation in plants. Pergamon Press, Oxford.

Wareing, P.F., J. Good, H. Potter and A. Pearson (1968). Soc. Chem. Ind. Monog. 31, 191-207.

Weber, J.A. (1972). Mich. Bot. 11, 115-121.

Weber, J.A. and L.D. Nooden (1976). Plant. Cell Physiol. 17, 721-732.

Weber, K. and M. Osborn (1975). In The Proteins, Vol. I (ed. H. Neurath and R.L. Hill), pp. 179-223. Academic Press.

Weibel, E.R. (1963). Morphometry of the Human Lung. Springer, Berlin-Gottingen-Heidleberg and Academic Press.

Weibel, E.R. (1973). In Principles and Techniques for Electron Microscopy, Vol. 3, Biological Apllications (ed. M.A. Hayat), pp. 238-296. Van Nostrand Reinhold.

Weibel, E.R., G.S. Kistler and W.F. Scherle (1966). J. Cell. Biol. 30, 23-38.

Weibel, E.R., W. Stäubli, H.R. Gnägi and F.A. Hess (1969). J. Cell. Biol. 42, 68-91.

Weinbaum, S.A., J. Gressel, A. Reisfield and M. Edelman (1979). Plant Physiol. 64, 828-832.

Weir, E.M., H. Riezman, J.-M. Grienenberger, W.M. Becker and C.J. Leaver (1980). Eur. J. Biochem. 112, 469-477.

Wellburn, F.A.M., A.R. Wellburn, J.L. Stoddart and K.J. Treharne (1973). Planta 111, 337-345.

Weyers, J.D.B. and J.E. Hillman (1979). Planta 144, 162-172.

Winston, R.D. and P.R. Gorham (1979). Can. J. Bot. 57, 2750-2759.

Witzum, A. (1974a). Amer. J. Bot. 61, 713-716.

Witzum, A. (1974b). Amer. J. Bot. 61, 805-808.

Witzum, A. (1979). Ann. Bot. 43, 423-430.

Witzum, A. and O. Keren (1978). New Phytol. 80, 111-115.

Witzum, A., H.B. Posner and R.A. Gower (1979). Ann. Bot. 44, 1-4.

Wołek, J. (1974a). Ver. Geobot. Inst. Eidg. Techn. Hochsch. Stift. Rübel. Zürich. 42, 140-162.

Wołek, J. (1974b). Ver. Geobot. Inst. Eidg. Techn. Hochsch. Stift. Rübel, Zürich 42, 163-170.

Wollgiehn, R. (1967). Symp. Soc. Exp. Biol. 21, 231-246.

Wood, A. and L.G. Paleg (1972). Plant Physiol. 50, 103-108.

Wright, S.T.C. (1961). J. Exp. Bot. 12, 303-318.

Wright, S.T.C. (1966). J. Exp. Bot. 17, 165-176.

Wright, S.T.C. (1975). J. Exp. Bot. 26, 161-174.

Wright, S.T.C. and R.W. Hiron (1969). Nature 224, 719-720.

Wright, S.T.C. and R.W. Hiron (1970). In Plant Growth Substances (ed. P.J. Carr), pp. 291-298. Springer Verlag, Berlin.

Wroblewski, R. (1973). J. Submicrosc. Cytol. 5, 97-105.

Wyen, N.V., S. Erdei, J. Udvardy, G. Bagi and G.L. Farkas (1972). J. Exp. Bot. 23, 37-44.

Zagórski, W. (1978). Eur. J. Biochem. 86, 465-472.

Zeevaart, J.A.D. (1971). Plant Physiol. 48, 86-90.

Zennie, T.M. and J.W. McClure (1977). Aquatic Botany 3, 49-54.

Zucker, M. (1972). Annu. Rev. Plant Physiol. 23, 133-156.

Zwar, J.A. and J.V. Jacobsen (1972). Plant Physiol. 49, 1000-1006.